# THE INFLUENCE OF THE ADRENAL GLANDS ON RESISTANCE.

## III. Susceptibility to Histamine as a Test of Adrenal Deficiency.*

By W. J. M. SCOTT, M.D.

*(From the Department of Surgery, the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)*

(Received for publication, November 17, 1927.)

The participation of the adrenal glands in the non-specific resistance of the animal has been previously shown. The clearest and most significant demonstration of this function is probably found in the case of bacterial intoxications, using either dead bacteria or their soluble toxins. The proper dose of the usual pyogenic organisms, killed by heat, seriously injured none of the operated controls but killed all of the doubly adrenalectomized rats (8). This increased susceptibility of adrenalectomized rats has been verified in the case of standard typhoid vaccine by Marine and Jaffe (4) and for diphtheria toxin by Belding and Wyman (2).

The resistance to such bacterial toxic agents is undoubtedly of major importance in pathological physiology. But, in studying quantitatively this function of the adrenal cortex, biological substances have the distinct disadvantage of variation, while a definite chemical compound with a somewhat similar pharmacological effect would be advantageous as a test substance. With this in view in 1923, while studying the effect of killed streptococci, the action of histamine was investigated in a few instances. Inasmuch as this substance seemed to possess the desired characteristics, the investigation of its effect has recently been studied in detail.

There is very little in the literature about the susceptibility of adrenalectomized animals to histamine. Dale (3) originally found

* Presented in abstract before the American Society for Experimental Pathology, Rochester, New York, April 14, 1927.

that cats, while still in good condition after adrenalectomy, were prostrated and killed by surprisingly small doses of this chemical. This finding was verified by Kellaway and Cowell (6). Banting has recently repeated this observation upon dogs (1). In both of these species, however, adrenalectomy results fatally within a few days. The present study reports the effect of histamine in the sublethal adrenal deficiency of rats.[1]

## *Methods.*

The plan of procedure was similar to that used in previous studies (7, 8). Rats (*Mus norvegicus albinus*) were kept in individual cages upon a standard diet and at a constant favorable temperature. After a period of observation they were operated upon,[2] both adrenals being removed from some, and one adrenal and a mass of fat from the site of the second adrenal were removed from others as a control upon the effect of the operation. The adrenals were removed by the dorsal route as being somewhat simpler than the ventral route used in the first study.

At determined intervals after operation the resistance of the rats to histamine injected intraperitoneally and intramuscularly was tested. The compound used was ergamine acid phosphate (Burroughs Wellcome and Co.) and all the dosages are reported on the basis of milligrams of ergamine acid phosphate crystals per 100 gm. weight of the animal. This contains about one-third of the amount of histamine base. The animals tested in this manner were all active and were eating well. Any showing clinical evidence of adrenal insufficiency were excluded. The chemical was usually given intraperitoneally 14 days after operation.

## *Results.*

The rat is ordinarily very resistant to histamine. I have not determined exactly the minimum lethal dose for my control animals due to the expense of the large amounts required. An operated control rat was apparently recovering from 110 mg. of ergamine acid phosphate per 100 gm. given in two doses 25 minutes apart and required another large dose 2 hours later to kill it. 30 mg. of ergamine acid phosphate per 100 gm. intraperitoneally was not fatal to four control rats and

[1] Since writing this report I have found a statement by Crivellari (Crivellari, C. A., *Am. J. Physiol.*, 1927, lxxxi, 414) that adrenalectomized rats have a greatly increased susceptibility to histamine.

[2] All operations were carried out under ether anesthesia.

killed one operated control rat that had recently survived an injection of peptone. In fact the amount of histamine that is required to kill normal and operated control rats is enormous. Voegtlin and Dyer (9) reported the minimum lethal intravenous dose of histamine phosphate to be 90 mg. per 100 gm. The mode of administration affects the outcome greatly, and undoubtedly the minimum lethal intraperitoneal dose is larger than when the substance is given intravenously. All of 12 operated control rats survived doses of 10 to 20 mg. of ergamine acid phosphate per 100 gm. For tissue study eight others were sacrificed in excellent condition, obviously recovering from the transient effects of the injection in the same dosage when the doubly adrenalectomized animals died or were moribund. In fact none of the normal or operated control animals were rendered comatose or nonreactive to stimulation by this dose. The maximum effect of it was transient respiratory difficulty and a quieting of the animal.

Of 36 doubly adrenalectomized rats all but two were killed by this dose (10 to 20 mg. per 100 gm.). In 28 of these the drug was given intraperitoneally, with fatal result in each instance. In addition to these seven other adrenalectomized rats, given the same amount of histamine intraperitoneally, were sacrificed for histological study in a moribund condition The only two doubly adrenalectomized rats to survive 10 mg. per 100 gm. of ergamine acid phosphate were given the drug intramuscularly. Six others injected in the latter manner were killed by 15 mg. per 100 gm. Two of the rats that as controls had survived 10 mg. per 100 gm. were later killed by the same dose after the second adrenal was removed. The exact time intervals after operation, the period of survival and the mode of injection may be determined from the tabulation (Table I). Most of the rats were injected intraperitoneally about 14 days after operation. All that were tested were in good condition and were indistinguishable in appearance and behavior from the operated control rats in adjoining cages. Most of them had lost a little weight after operation (averaging 5 per cent). Such was not the cause of the fatal effect, however, as the latter occurred even when the weight loss was absent.

The striking difference in susceptibility to histamine displayed by the adrenal deficient and the control animals is shown also in the effect of larger doses. Compare the result of injecting 60 mg. per 100

# THE INFLUENCE OF THE ADRENAL GLANDS ON RESISTANCE.

## III. Susceptibility to Histamine as a Test of Adrenal Deficiency.*

By W. J. M. SCOTT, M.D.

*(From the Department of Surgery, the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)*

(Received for publication, November 17, 1927.)

The participation of the adrenal glands in the non-specific resistance of the animal has been previously shown. The clearest and most significant demonstration of this function is probably found in the case of bacterial intoxications, using either dead bacteria or their soluble toxins. The proper dose of the usual pyogenic organisms, killed by heat, seriously injured none of the operated controls but killed all of the doubly adrenalectomized rats (8). This increased susceptibility of adrenalectomized rats has been verified in the case of standard typhoid vaccine by Marine and Jaffe (4) and for diphtheria toxin by Belding and Wyman (2).

The resistance to such bacterial toxic agents is undoubtedly of major importance in pathological physiology. But, in studying quantitatively this function of the adrenal cortex, biological substances have the distinct disadvantage of variation, while a definite chemical compound with a somewhat similar pharmacological effect would be advantageous as a test substance. With this in view in 1923, while studying the effect of killed streptococci, the action of histamine was investigated in a few instances. Inasmuch as this substance seemed to possess the desired characteristics, the investigation of its effect has recently been studied in detail.

There is very little in the literature about the susceptibility of adrenalectomized animals to histamine. Dale (3) originally found

* Presented in abstract before the American Society for Experimental Pathology, Rochester, New York, April 14, 1927.

that cats, while still in good condition after adrenalectomy, were prostrated and killed by surprisingly small doses of this chemical. This finding was verified by Kellaway and Cowell (6). Banting has recently repeated this observation upon dogs (1). In both of these species, however, adrenalectomy results fatally within a few days. The present study reports the effect of histamine in the sublethal adrenal deficiency of rats.[1]

## *Methods.*

The plan of procedure was similar to that used in previous studies (7, 8). Rats (*Mus norvegicus albinus*) were kept in individual cages upon a standard diet and at a constant favorable temperature. After a period of observation they were operated upon,[2] both adrenals being removed from some, and one adrenal and a mass of fat from the site of the second adrenal were removed from others as a control upon the effect of the operation. The adrenals were removed by the dorsal route as being somewhat simpler than the ventral route used in the first study.

At determined intervals after operation the resistance of the rats to histamine injected intraperitoneally and intramuscularly was tested. The compound used was ergamine acid phosphate (Burroughs Wellcome and Co.) and all the dosages are reported on the basis of milligrams of ergamine acid phosphate crystals per 100 gm. weight of the animal. This contains about one-third of the amount of histamine base. The animals tested in this manner were all active and were eating well. Any showing clinical evidence of adrenal insufficiency were excluded. The chemical was usually given intraperitoneally 14 days after operation.

## *Results.*

The rat is ordinarily very resistant to histamine. I have not determined exactly the minimum lethal dose for my control animals due to the expense of the large amounts required. An operated control rat was apparently recovering from 110 mg. of ergamine acid phosphate per 100 gm. given in two doses 25 minutes apart and required another large dose 2 hours later to kill it. 30 mg. of ergamine acid phosphate per 100 gm. intraperitoneally was not fatal to four control rats and

[1] Since writing this report I have found a statement by Crivellari (Crivellari, C. A., *Am. J. Physiol.*, 1927, lxxxi, 414) that adrenalectomized rats have a greatly increased susceptibility to histamine.

[2] All operations were carried out under ether anesthesia.

killed one operated control rat that had recently survived an injection of peptone. In fact the amount of histamine that is required to kill normal and operated control rats is enormous. Voegtlin and Dyer (9) reported the minimum lethal intravenous dose of histamine phosphate to be 90 mg. per 100 gm. The mode of administration affects the outcome greatly, and undoubtedly the minimum lethal intraperitoneal dose is larger than when the substance is given intravenously. All of 12 operated control rats survived doses of 10 to 20 mg. of ergamine acid phosphate per 100 gm. For tissue study eight others were sacrificed in excellent condition, obviously recovering from the transient effects of the injection in the same dosage when the doubly adrenalectomized animals died or were moribund. In fact none of the normal or operated control animals were rendered comatose or nonreactive to stimulation by this dose. The maximum effect of it was transient respiratory difficulty and a quieting of the animal.

Of 36 doubly adrenalectomized rats all but two were killed by this dose (10 to 20 mg. per 100 gm.). In 28 of these the drug was given intraperitoneally, with fatal result in each instance. In addition to these seven other adrenalectomized rats, given the same amount of histamine intraperitoneally, were sacrificed for histological study in a moribund condition The only two doubly adrenalectomized rats to survive 10 mg. per 100 gm. of ergamine acid phosphate were given the drug intramuscularly. Six others injected in the latter manner were killed by 15 mg. per 100 gm. Two of the rats that as controls had survived 10 mg. per 100 gm. were later killed by the same dose after the second adrenal was removed. The exact time intervals after operation, the period of survival and the mode of injection may be determined from the tabulation (Table I). Most of the rats were injected intraperitoneally about 14 days after operation. All that were tested were in good condition and were indistinguishable in appearance and behavior from the operated control rats in adjoining cages. Most of them had lost a little weight after operation (averaging 5 per cent). Such was not the cause of the fatal effect, however, as the latter occurred even when the weight loss was absent.

The striking difference in susceptibility to histamine displayed by the adrenal deficient and the control animals is shown also in the effect of larger doses. Compare the result of injecting 60 mg. per 100

TABLE I.

*Effect of Ergamine Acid Phosphate in Adrenalectomized and Control Rats.*

| Rat | | Operation | | Injection | | | | Result |
|---|---|---|---|---|---|---|---|---|
| No. | Sex | Weight | Removal of adrenals | Interval since operation | Weight | Method† | Dose | Period of survival in hrs. |
| | | *gm.* | | *days* | *gm.* | | *mg. per 100 gm.* | |
| B1 | ♀ | 212 | Left* | 81 | 212 | P | 20 | Survived |
| B2 | ♀ | 235 | Right* | 81 | 250 | P | 30 | 24 |
| B3 | ♀ | 216 | Right* | 67 | 216 | P | 10 | Survived |
| B4 | ♀ | 245 | Right* | 70 | 263 | P | 20 | Survived |
| B5 | ♂ | 180 | Right* | 23 | 189 | P | 20 | Survived |
| B7 | ♂ | 250 | Right* | 7 | 237 | P | 20 | Survived |
| R2 | ♂ | 300 | Right* | 12 | 308 | P | 10 | Survived |
| R3 | ♀ | 258 | Right* | 12 | 254 | P | 10 | Survived (*cf.* R3 later) |
| R5 | ♀ | 308 | Right* | 12 | 310 | P | 10 | Survived (*cf.* R5 later) |
| R8 | ♀ | 142 | Right* | 12 | 140 | P | 10 | Survived |
| R13 | ♂ | 198 | Right and spleen | 14 | 224 | M | 15 | Survived |
| R18 | ♂ | — | None | — | 294 | M | 30 | Survived |
| R20 | ♀ | 184 | Right* | 16 | 192 | M | 15 | Survived |
| R38 | ♂ | 220 | Right* | 4 | 220 | P | 60 | Recovering when sacrificed after 2 hrs. (*cf.* with R41) |
| R39 | ♂ | 228 | Perirenal fat | 4 | 220 | P | 10 | Survived |
| R40 | ♂ | 240 | Right* | 4 | 245 | P and M | 180 | Moribund after 45 min. |
| B6 | ♀ | — | Both | 21 | 205 | P | 15 | 1 |
| B8 | ♀ | 216 | Both | 7 | 208 | P | 20 | 2½ |
| B9 | ♀ | 177 | Both | 7 | 160 | P | 5 | 4½ |
| B10 | ♀ | 207 | Both | 7 | 205 | P | 10 | 1 |
| R1 | ♂ | 360 | Both | 12 | 324 | P | 10 | 35 min. |
| R3 | ♀ | 254 | Second | 7 | 230 | P | 10 | 2 (*cf.* R3 above) |
| R4 | ♀ | 218 | Both | 12 | 196 | P | 10 | 1¾ |
| R5 | ♀ | 310 | Second | 7 | 302 | P | 10 | 2 (*cf.* R5 above) |

* In most of the control operations when one adrenal was removed, the other adrenal was exposed and a piece of fat from its vicinity was removed to reproduce as completely as possible the physical conditions of the bilateral extirpation.

† In this column P signifies intraperitoneal and M signifies intramuscular.

TABLE I—*Concluded.*

| Rat | | Operation | | Injection | | | | Result |
|---|---|---|---|---|---|---|---|---|
| No. | Sex | Weight | Removal of adrenals | Interval since operation | Weight | Method† | Dose | Period of survival in hrs. |
| | | *gm.* | | *days* | *gm.* | | *mg. per 100 gm.* | |
| R6 | ♀ | 174 | Both | 12 | 160 | P | 10 | 40 min. |
| R7 | ♀ | 272 | Both | 12 | 256 | P | 10 | 1¾ |
| R9 | ♀ | 222 | Both | 12 | 202 | P | 10 | 40 min. |
| R10 | ♀ | 218 | Both | 12 | 194 | P | 10 | 2 |
| R11 | ♂ | 184 | Both | 12 | 206 | M | 10 | Survived |
| R12 | ♂ | 208 | Both | 12 | 224 | M | 10 | Survived |
| R14 | ♂ | 220 | Both | 13 | 216 | M | 15 | 2 |
| R15 | ♂ | 218 | Both | 13 | 212 | M | 15 | 2 |
| R16 | ♂ | 192 | Both | 13 | 190 | M | 15 | 1 |
| R17 | ♀ | 148 | Both | 12 | 138 | M | 15 | 25 min. |
| R19 | ♀ | 160 | Both | 17 | 154 | M | 15 | 2¼ |
| R21 | ♀ | 144 | Both | 16 | 136 | M | 15 | 4½ |
| R22 | ♂ | 264 | Both | 14 | 250 | P | 10 | 45 min. |
| R23 | ♂ | 278 | Both | 14 | 265 | P | 10 | 1¼ |
| R24 | ♂ | 234 | Both | 14 | 205 | P | 10 | 1¼ |
| R25 | ♂ | 250 | Both | 14 | 234 | P | 10 | 1¼ |
| R26 | ♂ | 232 | Both | 14 | 205 | P | 10 | 1¼ |
| R27 | ♂ | 280 | Both | 14 | 260 | P | 10 | 1¼ |
| R28 | ♂ | 242 | Both | 14 | 230 | P | 10 | 1¼ |
| R29 | ♂ | 244 | Both | 14 | 245 | P | 10 | 30 min. |
| R30 | ♂ | 278 | Both | 14 | 252 | P | 10 | 1 |
| R31 | ♂ | 256 | Both | 14 | 250 | P | 10 | 1¾ |
| R32 | ♂ | 262 | Both | 14 | 255 | P | 10 | 45 min. |
| R33 | ♂ | 212 | Both | 14 | 220 | P | 10 | 1 |
| R34 | ♂ | 224 | Both | 1 | — | P | 10 | 1 |
| R35 | ♂ | 202 | Both | 1 | — | P | 10 | 50 min. |
| R36 | ♂ | 210 | Both | 4 | 200 | P | 10 | 40 min. |
| R37 | ♂ | 242 | Both | 4 | 235 | P | 20 | 25 min. |
| R41 | ♂ | 246 | Both | 4 | 230 | P | 60 | 2 min. (*cf.* with R38) |

gm. in Rats R38 and R41, both active and in good condition before injection. The operated control animal was sacrificed 2 hours after the injection, apparently recovering from it, while the doubly adrenalectomized rat was killed by the histamine in 2 minutes.

Most doubly adrenalectomized rats, then, are killed by histamine in doses that produce only transient symptoms in operated control

rats and, in my experience, 10 mg. per 100 gm. of ergamine acid phosphate injected intraperitoneally has regularly proven fatal, if given in the 2nd week after bilateral adrenalectomy.

## DISCUSSION.

As the first corollary of this work I propose the reaction of rats to histamine injected intraperitoneally in the 2nd week after operation as one test of adrenal deficiency. While we have not directly proven that the function demonstrated in this diminished resistance to histamine is cortical, from its results viewed in the light of certain previous investigations this conclusion appears to be justified. Kellaway and Cowell (6) correlated the fatal effect of small doses of histamine in their operated cats with the destruction of the cortex and not with the loss of the medulla. Banting (1) found that adrenalin could not counteract the fatal effect of minute doses of histamine for adrenalectomized dogs. And Jaffe (5) demonstrated that autoplastic cortical transplants increase the diminished resistance of adrenalectomized rats to the toxic effect of killed bacteria. Thus, in correlating these findings with our data it appears that the function involved is cortical and is not related to adrenalin deficiency.

Physiological studies of the adrenal cortex have been handicapped by the fact that there has been no definite simple criterion of its function in any but the terminal phases. This has made extremely difficult the important search for a substitution product to compensate for lost cortical function. Changes in the blood chemistry of these animals, while striking in the terminal stages, are not sufficiently delicate or constant in the period of well being of the animal to serve as an index of the effect of any procedure upon the adrenal deficiency. While less desirable than a chemical index that could be followed in the same animal, the reaction of adrenalectomized rats to histamine offers a method of determining the substitutive effect of a procedure for that adrenal function which influences the non-specific resistance of the animal. At the present time this test is being used in a study of the efficacy of adrenal cortex extracts.

During the course of this study interesting abnormal responses on the part of the peripheral vascular system in adrenal insufficiency have been observed. These will be reported in a separate paper.

## CONCLUSIONS.

1. The resistance of rats to histamine is greatly diminished after adrenalectomy.

2. This susceptibility to histamine is proposed as a functional test for deficient adrenal cortex function.

## BIBLIOGRAPHY.

1. Banting, F. G., and Gairns, S., Suprarenal insufficiency, *Am. J. Physiol.*, 1926, lxxvii, 100.
2. Belding, D. L., and Wyman, L. C., The rôle of the suprarenal gland in the natural resistance to diphtheria toxin, *Am. J. Physiol.*, 1926, lxxviii, 50.
3. Dale, H. H., Conditions which are conducive to the production of shock by histamin, *Brit. J. Exp. Path.*, 1920, i, 103.
4. Jaffe, H. L., and Marine, D., Effect of suprarenalectomy in rats on agglutinin formation, *J. Infect. Dis.*, 1924, xxxv, 334.
5. Jaffe, H. L., On diminished resistance following suprarenalectomy in the rat and the protection afforded by autoplastic transplants, *Am. J. Path.*, 1926, ii, 421.
6. Kellaway, C. H., and Cowell, S. J., On the concentration of the blood and the effects of histamin in adrenal insufficiency, *J. Physiol.*, 1922, lvii, 82.
7. Scott, W. J. M., The influence of the adrenal glands on resistance. I. The susceptibility of adrenalectomized rats to morphine, *J. Exp. Med.*, 1923, xxxviii, 543.
8. Scott, W. J. M., The influence of the adrenal glands on resistance. II. The toxic effect of killed bacteria in adrenalectomized rats, *J. Exp. Med.*, 1924, xxxix, 457.
9. Voegtlin, C., and Dyer, H. A., Natural resistance of albino rats and mice to histamine, pituitary and certain other poisons, *J. Pharmacol. and Exp. Therap.*, 1924, xxiv, 101.

# STUDIES IN THE COMMON COLD.*

## II. A Study of Certain Gram-Negative Filter-Passing Anaerobes of the Upper Respiratory Tract.

By KATHERINE C. MILLS, G. S. SHIBLEY, M.D., and A. R. DOCHEZ, M.D.

(*From the Department of Medicine of the College of Physicians and Surgeons, and the Presbyterian Hospital, New York.*)

(Received for publication, November 11, 1927.)

The common cold is generally conceded to be of infectious origin. Attempts to assign the causative rôle to the bacteria more readily found in the upper respiratory tract during infection or in health have been uniformly unsuccessful. Such organisms have included, *Micrococcus catarrhalis*, *B. pfeifferi*, pneumococcus, streptococcus, staphylococcus, diphtheroids, certain anaerobes and various other bacteria. It is perfectly clear that several of these, such as, hemolytic streptococcus, *B. pfeifferi*, *Staphylococcus aureus* and pneumococcus, play an important part in the later phases of infection or in complications, but the actual inception of the cold would seem to be due to an organism or organisms as yet unknown. Much favorable evidence has been put forward (chiefly by transmission experiments) to implicate a filter-passing virus (Kruse (1), Foster (2), Olitsky and McCartney (3)); other observers, however, have obtained unconvincing results (Schmidt (4), Williams *et al.* (5), Branham and Hall (6), Robertson and Groves (7)).

Olitsky and McCartney (3), in their work with cold cultures, grew certain filter-passing anaerobes, previously described by Olitsky and Gates (8), to which they preferred to assign no etiological rôle in view of their occasional appearance in healthy normal individuals.[1] It was hoped that a careful study of the incidence and biological character of these new organisms might provide a clue to the solution of the

* This study was made possible by a grant from the Chemical Foundation, Inc.

[1] Branham (9) in a recent study of the occurrence of anaerobes in normals, influenza, colds, etc., has isolated and studied a number of these organisms.

problem. These organisms, as originally described by Olitsky and Gates, and further studied by Olitsky and McCartney, are anaerobic, Gram-negative bacilli, which pass through Berkefeld V and N filters, and which are culturally and serologically distinct from *B. pneumosintes*.

It has been pointed out in a previous paper (10), that familiarity with the bacterial flora of the upper respiratory tract in health is a prerequisite to correct interpretation of findings in the course of infection. Accordingly, during the first part of the investigation, a group of healthy normals was followed for a period of several months to determine the presence of these filter-passing anaerobes; during the same period search for the organisms was carried out in these subjects when they acquired colds and in many other individuals suffering from typical colds. During the 2nd year, less emphasis was placed upon the study of normals and much time was given to determining the occurrence of these organisms in persons with typical colds. Throughout the work many of the organisms, from both normals and cold cases, were studied culturally and serologically in an endeavor to demonstrate "normal" or "cold" strains.

## *Methods.*

*Material.*—In the winter of 1925–26, five healthy normal individuals were studied over a period of 5 months for the presence in their nasopharynges of Gram-negative, filter-passing anaerobes of the type noted above. During this period six colds occurred in the group and were similarly observed. At the same time nasal washings from twenty-seven other individuals suffering from typical colds were studied. In the winter of 1926–27, three normals were followed more or less continuously, and material obtained from sixty-seven cold cases for study; fifty-five of these colds occurred in members of the office staff of the American Telephone and Telegraph Company,[2] and the remainder in hospital personnel, students and patients.

*Time of Cultures.*—In the normals studied, cultures were made weekly at first and later at 2 to 3 week intervals. In cold cases, cultures were single ones, taken within the first 24 hours of infection.

*Method of Obtaining Culture Material.*—The material for culture consisted of washings obtained by running various solutions (warmed) gently into the nose

[2] Permission to make use of the abundant clinical material in this large group of individuals was very kindly granted us by Dr. Cassius Watson of the American Telephone and Telegraph Company.

(5 cc. per nostril) and thence out through the mouth: in addition, subjects usually added 10 to 15 cc. by gargling. During the 1st year of the work, stock buffered broth (pH 7.6) was used for the washings. When the study was extended to the American Telephone and Telegraph Company group, Ringer's solution, as recommended by Olitsky and McCartney (3), was substituted because of the disagreeable taste of the broth. Our results with this fluid were so rarely positive that after a few weeks we returned to the use of broth. Both Ringer's solution and physiological salt solution seem to be bactericidal for these delicate organisms, and successful culture is much more likely when broth is used in washing out the nasopharynx as survival during the period of filtration and transfer is enhanced.

TABLE I.

*Incidence of Gram-Negative Anaerobic Organisms in a Normal Subject (F. M. H.) on Seven Successive Cultures.*

| No. | Date | Culture media | | | | |
|---|---|---|---|---|---|---|
| | | Blood plate | Smith-Noguchi | *B. coli* broth | Dextrose broth and kidney | Dextrose broth and kidney, aerobic or blood broth |
| 1 | 11–11–25 | − | + | − | − | − |
| 2 | 11–25–25 | + | − | − | − | − |
| 3 | 12– 7–25 | − | − | + | − | − |
| 4 | 12–21–25 | − | − | + | − | − |
| 5 | 1– 4–26 | − | − | − | − | − |
| 6 | 1–18–26 | − | − | + | + | + |
| 7 | 2– 2–26 | + | − | − | + | + |

+ = growth.

*Cultural Methods.*—The nasal washings thus obtained were collected in sterile bottles and thoroughly shaken with glass beads. Some of this material was inoculated upon fresh 5 per cent rabbit's blood agar plates and incubated aerobically.

The remainder of the material was passed through Berkefeld V filters. About 0.5 cc. of the filtrate was seeded routinely in various media, as follows: 1925–26, Smith-Noguchi medium, as modified by Gates and Olitsky (11), *B. coli* broth (12), *fresh*, moist, 5 per cent rabbit's blood agar plates and dextrose broth; 1926–27, Smith-Noguchi media, *fresh* rabbit's blood agar plates and rabbit's blood broth. Cultures were incubated anaerobically, anaerobiosis being obtained with the Brown modification (13) of the McIntosh and Fildes (14) jar. Plates were examined after 1 week. Fluid cultures were plated after 1 week, in the case of Smith-Noguchi media plating was repeated after 2 weeks. Study of each case, therefore, took at least 2 to 3 weeks. The reason for the multi-

plicity of medium inoculations noted above lay in the fact that the growth of these delicate organisms is so uncertain that dependence upon one medium alone leads to many negative results although organisms are actually present in the washings. The following record of seven successive cultures in one of the normals studied in 1925–26 illustrates this point, Table I. It will be seen from this table that the incidence of positive single cultures in the complete series is eleven out of thirty-five (31 per cent); actually the washings were positive in six of the seven attempts to recover the organisms, a real percentage incidence of 86 per cent.

During the 2nd year, as noted above, three culture media per washing were deemed sufficient; in our 1st year's experience we had found that positive results were more frequently obtained with the use of blood plates, blood broth and Smith-Noguchi media. An additional reason for our use of the last named culture medium lay in the fact that we were particularly interested in the possible incidence of *B. pneumosintes*, and it has been emphasized by Olitsky and Gates (12), and Gates and Owens (15) that primary cultures of this organism are obtained on this medium only.

Additional cultural methods that were used in an effort to grow the organisms more easily, more surely or in profusion, and which were discarded as unsatisfactory or inferior included inoculation of potato broth, blood agar, pH 7.2, plain broth, sugar broth and blood agar to which filtered fresh tomato juice had been added, agar blebs in rats and chick embryos (16).

For determining sugar fermentations, chocolate broth with 1 per cent of various sugars tested was used. Preparation was as follows: NaCl meat infusion broth, pH 7.2, plus 4 per cent rabbit's blood was heated to 90°C., filtered through paper, and after addition of sugar (1 per cent) was passed through Berkefeld V filters and tubed. After inoculation, tubes were incubated 1 week and any changes in pH determined colorimetrically. The broth in each case was plated and reincubated for check upon growth or survival of the organisms.

*Motility.*—This was determined by dark-field illumination. There was marked Brownian movement in all cultures tested. Organisms were considered to be motile only when definite progressive motion was observed.

*Serological Methods.*—Agglutinating sera were prepared by injecting rabbits with live cultures. Three successive daily injections per week for 6 weeks were planned. The marked difficulty, however, of obtaining uniform quantities of the organisms at the times needed led to occasional departures from this routine. Agglutination reactions were done in the water bath at 56°C. (2 hours); readings were made after leaving the tubes in the ice box overnight. Dilutions were carried up to 1:320 whenever sufficient organisms were available. Absorption experiments were not undertaken because of the difficulty in growing sufficient organisms; moreover, cross-agglutination was so infrequent that use of this method was rarely indicated.

*Incidence of Gram-Negative Anaerobes.*

*Incidence in Normals.*—A summary of the results of attempts to isolate Gram-negative filter-passing anaerobes from normals in 1925–26 is shown in Table II. During this period broth only was used for nasal washings. The percentage incidence for the group is 78.6 per cent. Case 1 shows a low incidence, 50 per cent. The organisms were recovered from the other individuals at nearly every examination.

TABLE II.

*Incidence of Gram-Negative Anaerobic Organisms in Normals, Winter of 1925–26.*

| Subjects | No. of examinations | Positive cultures | Incidence |
|---|---|---|---|
| | | | *per cent* |
| K. C. M. | 14 | 7 | 50 |
| F. M. H. | 7 | 6 | 86 |
| R. F. L. | 3 | 3 | 100 |
| E. R. | 3 | 3 | 100 |
| G. S. S. | 15 | 14 | 94 |
| Totals | 42 | 33 | 78.6 |

TABLE III.

*Incidence of Gram-Negative Anaerobic Organisms in Normals, Winter of 1926–27.*

| Subjects | No. of examinations | Positive cultures | Incidence |
|---|---|---|---|
| | | | *per cent* |
| K. C. M. | 7 | 6 | 85.7 |
| S. | 2 | 1 | 50 |
| G. S. S. | 6 | 4 | 66.6 |
| Miscellaneous | 5 | 3 | 60 |
| Totals | 20 | 14 | 70 |

In 1926–27 washings were first made with Ringer's solution, and during this period (about 2 months) the percentage incidence was only 21.4 per cent. We therefore discarded this method and returned to the use of broth. This change proved to be justified as we now obtained a 70 per cent incidence, a result comparable to the findings of the 1st year, Table III.

The net incidence therefore of these organisms, in normals, without

consideration of their type or of pathogenicity, is quite high. When one remembers the extreme difficulty of their cultivation it seems fair to assume that these organisms constitute part of the normal flora of the nasopharynx. That their habitat is the nasopharynx was shown by our ability to recover the organisms regularly from gargled broth and our failure to cultivate them from teeth and gums.

*Incidence in Colds.*—In 1925–26 six colds occurred in the normal group; Gram-negative filter-passing anaerobes were recovered from half of these. During the year, twenty-seven other colds were cultured with thirteen positives. The percentage incidence for the year was 48.5 per cent. Broth was used for all washings.

TABLE IV.

*Comparison between Incidence of Gram-Negative Anaerobic Organisms in Normals and in Colds, 1925–27.*

| Year | Normals | | | Colds | | |
|---|---|---|---|---|---|---|
| | No. of examinations | Positive cultures | Incidence | No. of examinations | Positive cultures | Incidence |
| | | | *per cent* | | | *per cent* |
| 1925–26 | 42 | 33 | 78.6 | 33 | 16 | 48.5 |
| 1926–27 | 20 | 14 | 70 | 9 | 4 | 44.4 |
| Totals..... | 62 | 47 | 75.8 | 42 | 20 | 47.4 |

During 1926–27 sixty-seven colds were studied. Ringer's solution was used for washings in the first twenty-seven with only one positive result. We therefore went back to the use of broth as in the work with normals. Following this change, local colds showed the organisms again in about the percentage noted for the previous year, 44.4 per cent. In the American Telephone and Telegraph Company group however, the incidence remained quite low (9.4 per cent). Our failure to recover the organisms from these cases may well be attributed to the fact that cultures were transported over considerable distances and that the time elapsing between making the washings and filtration and cultural procedure often ran to 2 or 3 hours.

Table IV shows a summary of the findings in colds and a comparison between these results and those obtained in normals.

In normals, the percentage incidence was based upon several cultures per individual, while one culture per person was the rule in colds. That this was not a source of error in comparing the two groups is shown by the fact that the percentage incidence in normals, obtained by using the results of their first cultures only, was 80 per cent in the 1st year and 75 per cent in the 2nd.

Cultures in which Ringer's solution was used for washings have been purposely omitted as have the American Telephone and Telegraph Company colds, because conditions here were not comparable. If one leaves out of consideration possible variations in strain, and notes only changes in gross incidence, it will be seen at once that Gram-negative anaerobes of the type under consideration appear much less often in colds than under normal conditions. As will appear shortly, we have been able to produce no evidence in favor of the fact that such types as "cold strains" exist. Hence we are justified in concluding that there is a definite reduction in the incidence of these anaerobes in the course of colds, a thing hardly to be expected if these organisms bear an etiological relationship to colds. It is worthy of note that we have demonstrated in a previous paper (10) a similar quantitative reduction, during colds, of most of the organisms that are commonly considered to be normal non-pathogenic inhabitants of the nose and throat, *i.e.*, *Staphylococcus albus*, diphtheroids, Gram-negative cocci, etc.

Attention should be called to the fact that at no time in our work with normals and with colds (we had no cases of influenza) did we recover typical *B. pneumosintes*. We made every effort to cultivate the organisms except the use of animal transmission, and followed carefully all the dicta of Gates and Olitsky (12) and Gates and Owens (15). Also none of our Gram-negative anaerobes agglutinated with the one *B. pneumosintes* serum we were able to prepare.[3] The importance of this negative observation lies in the fact that Olitsky and Gates have stated that this organism is never found in normal throats and appears only in influenza.

[3] Culture of *B. pneumosintes* (C17, isolated April 10, 1918) kindly given to us by Dr. Olitsky of The Rockefeller Institute, in July, 1925.

*Cultural and Biological Characteristics.*

*Material for Study.*—During the course of the investigation, Gram-negative anaerobic bacilli were recovered seventy-six times. From these, twenty-nine strains were preserved for detailed study; of this group eighteen were from normals and eleven from colds. Most of the collected strains were obtained during the 2nd year of the work. The strain of *B. pneumosintes* referred to above was added to the group for parallel study. All but three of the strains were obtained by routine inoculation of the media enumerated above with filtrates from washings. In the case of the exceptions, unfiltered washings were injected into agar blebs in the abdominal walls of rats, and the organisms recovered in two cases from the bleb and in the third from the heart and lungs. These strains were never passed successfully through filters, but morphologically and culturally they were identical with the organisms of the group, therefore they were included for study.

Olitsky and Gates (8) and Olitsky and McCartney (3) have divided the organisms into three definite types (I, II, III) on the basis of morphological, cultural and serological differences. Our results, as will be noted below, although showing rather looser groups, are in general agreement except for our serological findings. In the description that follows we have made use of their classification.

*Morphology and Staining.*—The organisms are extremely variable in appearance. The chief forms encountered were minute coccobacilli, short fairly straight delicate bacilli (smaller than *B. pfeifferi*), slender curved comma bacilli and long slender spirilliform bacilli. The range in length was from 0.2 to 6.0 micra. Of these, the coccobacilli usually remained true to type (Group II) while the remaining strains (Groups I and III) frequently appeared in one or more of the other forms. In Group I the most distinctive type was the comma bacillus although some of the strains in this group never showed this form. In Group III the most distinctive type was a small delicate straight bacillus. The organisms decolorize easily by Gram's method. Dilute carbol fuchsin was found to be the best counterstain. There was occasional irregular staining in the longer forms, giving a beaded appearance to the organisms.

*Motility.*—Twelve of the twenty-nine strains showed motile forms. We were unable to demonstrate any relationship between motility and type.

*Filtrability.*—All strains except the three noted above were recovered from filtrates of washings passed through Berkefeld V candles. Attempts to pass the

organisms through filters after we had them growing failed in all instances but one, and in this case we were unable to confirm the result because of contamination of the culture. It is of special interest to note that in two out of three attempts, passage through chick embryos restored filtrability of the organisms.

*Cultural Characteristics.*—The organisms are strictly anaerobic. They will survive, however, for many days upon plates or in broth under aerobic conditions.

*Growth in Fluid Media.*—A certain number of the strains usually grew readily in fluid media (I). Others were characterized by the great difficulty of their cultivation in this medium (II, III). Growth was always diffuse. Of the media used, blood broth gave the most satisfactory results, *B. coli* broth was next and Smith-Noguchi medium least useful. Our strain of *B. pneumosintes* grew very well in these media.

*Growth on Solid Media.*—Growth on blood agar plates was generally satisfactory, although often scanty and rarely profuse. On several occasions however, we observed very luxuriant growth on some of the plates. We made many attempts to find a cause for this unusual growth, but could not account for it, although we were able to demonstrate that it was not a symbiotic phenomenon. Blood agar plates were much the most satisfactory for preservation of stock strains. Growth on tomato juice blood agar was very good; it was poor upon chocolate agar. That the organisms are not strict hemoglobinophiles was shown by their capacity for moderate growth on plain agar plates. On blood plates there was slight hemolysis at times, most marked in the case of the coccobacillary type.

Colony formation on blood agar plates was very variable, except in the case of the coccobacillary group (II) which grew uniformly as smooth translucent dewdrop colonies, quite similar to *B. pneumosintes* but smaller. A second group (I) appeared often as fine, opaque, conical colonies with smooth edges; they frequently showed variations however, which included granular consistency, rough edges or rounded tops. A third group (III) was entirely inconsistent as to colony formation; its most frequent appearance was as a flat, slightly granular, opaque colony with irregular edges and raised center, other forms included small smooth, large granular rounded, or large smooth brown colonies with metallic sheen, the last often leaving a brown area on scraping the plate. One organism, NSh5, morphologically III, fell into none of these groups; for 2 years it showed fairly consistently flat, granular, translucent colonies with irregular edges. The first and last mentioned types constituted a very small proportion of the whole group studied; accordingly colony formation seems to be an uncertain criterion for classification of the organisms.

*Sugar Fermentations.*—The paucity of growth of these organisms in fluid media made determination of their sugar fermentations difficult; absence of sugar splitting may occasionally have been due to scanty growth. Fairly definite conclusions have been arrived at by repeated experiments.

Sugars used were dextrose, saccharose, galactose and levulose. Of the twenty-nine strains studied, eight fermented dextrose; the usual final pH was 5.2; these

TABLE V.

*Summary of Agglutination Reactions with Six Representative Sera and One B. pneumosintes Serum.*

| Strains tested | | Agglutinating sera | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Type I | | | Type II | Type III | | *B. pneumosintes* |
| | | NMc | CH | CLA1 | NDs | NSh5 | CW | |
| Group I | NM4 | CCCCC3* | ------ | ------ | ------ | ------ | ------ | ------ |
| | NM13 | CCCCC– | ------ | ------ | ------ | ------ | ------ | ------ |
| | NM15s | | CC2--- | | | | ------ | ------ |
| | NMc | CCCCC3 | ------ | ------ | ------ | ------ | ------ | ------ |
| | CE3 | | ------ | | | | | ------ |
| | CH | ------ | CCCC21 | ------ | ------ | ------ | ------ | ------ |
| | CLA1 | ------ | ------ | CCCCC– | | ------ | ------ | ------ |
| | CLB | | | | | | | ------ |
| | CMx | ------ | ------ | ------ | ------ | ------ | ------ | ------ |
| Group II | NDs | | | | 22– | | | |
| | NMR | | | | CCC | | | |
| | NSS | | | | | | | |
| | NM9 | | | | | | | |
| | CM2b | | | | | | | |
| Group III | NDL | | | | | | | ------ |
| | NM1 | | | | | ------ | ------ | ------ |
| | NM2 | | ------ | | | | CCC2-- | ------ |
| | NM10 | | | | | | ------ | ------ |
| | NM151 | ------ | ------ | | | | ------ | ------ |
| | NS1 | | | | | | | ------ |
| | NSh1 | ------ | ------ | ------ | ------ | ------ | ------ | ------ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Group III | NSh5 | ------ | ------ | ------ | ------ | CCCCC3 | ------ | ------ |
| | NSh9 | | | | | ------ | C22--- | ------ |
| | NSh12 | | | | | | | |
| | CM1 | | | | | | | ------ |
| | CM2a | ------ | ------ | | | | ------ | ------ |
| | CO | | 1----- | | | | ------ | 1----- |
| | CSh | ------ | ------ | ------ | ------ | ------ | ------ | ------ |
| | CW | ------ | ------ | | | ------ | CCCCC3 | ------ |
| *B. pneumosintes* | | ------ | ------ | ------ | ------ | ------ | ------ | CCCCC2 |

*Agglutination dilutions above; 1/10, 1/20, 1/40, 1/80, 1/160, 1/320.

C = complete agglutination, supernatant fluid clear; 3 = same, fluid not quite clear; 2 = gross granular appearance without settling; 1 = fine granular appearance; – = no agglutination.

strains also fermented the other three sugars. *B. pneumosintes* which was tested also split all four sugars. The remaining twenty-one strains failed to ferment any of the sugars tested. The first named eight strains fell into Group I.

*Serological Characteristics.*—Preparation of the agglutinating sera was beset with many difficulties as noted above. Six sera in all were made; three of these with Group I organisms (two cold strains and one normal), one with a Group II strain (normal) and two with Group III organisms (one cold and one normal strain). A *B. pneumosintes* serum was prepared for parallel tests. We were unable to determine the titre of the Group II serum as we failed consistently to cultivate enough of the homologous organisms to perform satisfactory agglutination reactions.

Table V shows the results. Organisms are arranged according to their morphological and cultural classification. Strains obtained from normals are indicated by the strain symbol N and those from colds by a C. In Group I there were four strains from normals and five from colds, in Group II, four from normals and one from a cold, and in Group III, ten from normals and five from colds.

Reference to the findings in the table shows that there is very little evidence of serological homogeneity in "cold" or "normal" strains or in the three cultural groups. No cross-agglutination occurred between "cold" strains either in or out of the cultural groups. Twice only there was agglutination of "normal" strains by "normal" sera; NM4 and 13 (from same person) by serum NMc (Group I) and NMR by serum NDs (Group II). Twice "normal" strains were agglutinated by "cold" strains: NM15s by serum CH (Group I) and NM2 and NSh9 by serum CW (Group III). Agglutination in Group II was most unsatisfactory for the reasons referred to above; as most of these organisms came from normals and as our principle endeavor was to demonstrate "cold strains," this lack is of little importance. Except for the occasional cross-agglutination noted above we were unable to confirm the findings of Olitsky and McCartney that strains in the cultural groups are serologically related. There was no agglutination of "normal" or "cold" strains by the *B. pneumosintes* serum.

*Summary.*—Our observations regarding this group of organisms with respect to their morphological and cultural characteristics and classification are in general accord with those of Olitsky and Gates and Olitsky and McCartney, although we have noted rather wide variations in Groups I and III. Contrary to the findings of these authors our strains could not be grouped serologically. Although we have been able to divide the organisms into three classes we have been impressed by the heterogeneous character of the group.

## DISCUSSION.

Three outstanding facts appear from this study. (1) The organisms in question are a heterogeneous group, (2) they are nearly always present in the upper respiratory tract and (3) there is a decrease in their incidence during colds.

The lack of homogeneity among the strains was striking. Variations in morphological and cultural characteristics were the rule, even in the subgroups, and very little serological interrelationship was evident. We were unable to demonstrate either "cold" or "normal" strains. In regard to their heterogeneity these organisms resemble such poorly differentiated non-pathogens as *B. coli* or perhaps *B. pfeifferi*. It is true that virulent strains are sometimes noted among such organisms and it may be possible that such pathogenic strains exist among these Gram-negative filter-passing anaerobes, but we were able to produce no evidence in support of this.

The organisms seem to be part of the harmless normal flora of the upper respiratory tract. Probably their percentage incidence would rise higher than that noted by us (75 per cent) if cultural methods could be further improved. The reduction in their incidence in colds makes improbable the assumption that they bear a causal relationship to these infections. In fact, as pointed out above, this decrease is much more in keeping with the conclusion that they constitute part of the normal non-pathogenic flora of the upper respiratory tract.

## CONCLUSIONS.

1. A study of the Gram-negative, filter-passing, anaerobic organisms, described by Olitsky and Gates, and Gates and McCartney, has been undertaken with a view to determining their general character and their possible rôle in the causation of the common cold.

2. These organisms seem to constitute part of the normal flora of the upper respiratory tract and would seem to bear no etiological relationship to the common cold.

We wish to acknowledge our indebtedness to Dr. Cassius H. Watson of the American Telephone and Telegraph Company and his professional staff for assistance in obtaining nasal washings from colds.

BIBLIOGRAPHY.

1. Kruse, W., *Münch. med. Woch.*, 1914, lxi, 1547.
2. Foster, G. B., Jr., *J. Am. Med. Assn.*, 1916, lxvi, 1180; *J. Infect. Dis.*, 1917, xxi, 451.
3. Olitsky, P. K., and McCartney, J. E., *J. Exp. Med.*, 1923, xxxviii, 427
4. Schmidt, P., *Deutsch. med. Woch.*, 1920, xlvi, 1181.
5. Williams, A. W., *et al.*, *J. Immunol.*, 1921, vi, 5.
6. Branham, S. E., and Hall, I. C., *J. Infect. Dis.*, 1921, xxviii, 143.
7. Robertson, R. C., and Groves, R. L., *J. Infect. Dis.*, 1924, xxxiv, 400
8. Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.
9. Branham, S. E., *J. Bact.*, 1927, xiii, 3; *J. Infect. Dis.*, 1927, lxi, 203.
10. Shibley, G. S., Hanger, F. M., and Dochez, A. R., *J. Exp. Med.*, 1926, xliii, 415.
11. Smith, T., *J. Boston Soc. Med. Sc.*, 1898–99, iii, 340. Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99. Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51, 713.
12. Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.
13. Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.
14. McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.
15. Gates, F. L., and Owens, W., *J. Exp. Med.*, 1926, xliv, 787.
16. Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.

# STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

## IV. The Course of a Second Type of Salmonella Infection Naturally Appearing in the Endemic Stage.

By JOHN B. NELSON, Ph.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, November 26, 1927.)

The appearance of a second type of Salmonella in a guinea pig population during the endemic stage of a spontaneous outbreak of paratyphoid disease was reported in an earlier paper (1). The earliest cases of the first infection occurred in the summer of 1924. The associated organism became rapidly disseminated throughout the population; particularly through the breeding stock, and for a period of 8 weeks a moderately high specific death rate ensued. The epidemic subsequently declined to an endemic level which has persisted up to the present. The course of the infection was followed by a bacteriological examination of all animals that died in the population under observation.

During July and August, 1926, several strains of a second type of Salmonella were isolated from fatal cases. Subsequently there was a slow but general spread of the second organism. These natural occurrences, among the guinea pigs, afforded an opportunity for studying the course of a second infection in an animal population which had been exposed for a considerable period of time to a related but serologically distinct type of organism.

A somewhat similar condition in a mouse population was described by Lynch (2). A spontaneous outbreak of paratyphoid infection, of 2 years duration, was followed by a second outbreak caused by a serologically different type of Salmonella. The two types of organism were designated Mouse Typhoid I and II. Some 18 months prior to the second outbreak half of the population was vaccinated with a strain of Mouse Typhoid I. The specific death rate for the

ensuing 7 months was low. There followed a period of fluctuation, with the death rate in general on a higher level, which terminated in a sharp rise. At this time the second type of organism was isolated.

Later, Pritchett (3) encountered a reverse replacement during the course of an artificially induced mouse epidemic. This was started by placing normal individuals in contact with mice which had received *per os* injection of Mouse Typhoid II. After an interval of several months the original Mouse Typhoid II strain was almost entirely replaced by Mouse Typhoid I. The latter type was known to be mildly endemic in the breeding stock and was thought to have been introduced into the particular population by the chance addition of fecal carriers.

The recovery of a second type of Salmonella from mice under similar circumstances was reported by Topley (4). An epidemic was started by feeding broth cultures of *Bacillus Gaertner*. Some of the mice which died during the early stage of the epidemic yielded pure cultures of a different Salmonella type. The organism showed a close serological relationship to *Bacillus suipestifer* (mutton). In a later stage of the epidemic both types were frequently isolated from the same animal. It should be added that the earlier type corresponded with Mouse and Guinea Pig Type I, and the second organism with Type II.

It may be pertinent to refer, in passing, to the relationship of the mouse and guinea pig types of organism and to their position within the Salmonella group. It was previously shown that Mouse Typhoid I and Guinea Pig Paratyphoid I of our epidemic were intimately related if not identical strains of the same Salmonella type (5). A similar relationship was established for Mouse Typhoid II and Guinea Pig Paratyphoid II. That the latter are an *aertrycke* form of *Bacillus paratyphi* likewise appears established (1). The relationship of the former, the initial strain, to a distinct Salmonella type is less definitely indicated. Amoss and Haselbauer (6) have related Mouse Typhoid I to *Bacillus enteritidis*. For, of three *Bacillus enteritidis* strains, the first was agglutinated to the titer limit by a Mouse Typhoid I antiserum, the second to less than 50 per cent and the third not at all. Upon absorption the first strain reduced the agglutinin content 50 per cent, the second 60 per cent and the third anomalously 50 per cent. A similar relationship, on the basis of direct and reciprocal absorption tests with specific agglutinating serums, was indicated by Sakai (7). The absorption tests, however, revealed a considerable variation in the absorptive capacity of the strains employed. Nelson and Smith (5) reported only a remote relationship between the two rodent strains and two strains of *Bacillus enteritidis*. A specific antiserum which agglutinated the former in a dilution of 1:51,200 agglutinated the latter in a dilution of 1:800 and 1:400 respectively. Absorption tests were not run. There appears to be nearly as wide a variation between different strains of *Bacillus enteritidis* and between the same and the Type I rodent strains as there is between the latter and the Type II rodent strains.

A general discussion of the rodent forms of the paratyphoid-enteritidis group is given by Jordan (8). He divided them into two main types, one related to *Bacillus enteritidis*, the other to an *aertrycke* form of human *Bacillus paratyphosus*.

In the several papers of the present series the two organisms under discussion have been consistently desginated as *Bacillus paratyphi* of a given type, I or II. This broad designation has been adopted as a matter of convenience pending more satisfactory evidence for the species relationship of the initial form.

The course of the Type II infection is considered first with reference to its percentage mortality within the population at large and secondly with reference to its cage to cage spread among the breeding stock. The former is computed from the specific deaths occurring within the total estimated population by months. The figures are only approxi-

TABLE I.

*Population, Total Deaths, Deaths from Paratyphoid and Percentage Mortality from Paratyphoid, July, 1925, through June, 1926.*

| Month | Population | Total deaths | Deaths from paratyphoid | Mortality from paratyphoid |
|---|---|---|---|---|
| | | | | *per cent* |
| July | 401 | 51 | 10 | 2.49 |
| August | 460 | 8 | 5 | 1.08 |
| September | 438 | 27 | 16 | 3.65 |
| October | 500 | 7 | 5 | 1.00 |
| November | 540 | 9 | 3 | 0.55 |
| December | 581 | 15 | 0 | 0.00 |
| January | 470 | 22 | 1 | 0.21 |
| February | 455 | 31 | 11 | 2.41 |
| March | 493 | 24 | 1 | 0.20 |
| April | 491 | 17 | 2 | 0.40 |
| May | 502 | 18 | 0 | 0.00 |
| June | 478 | 33 | 7 | 1.46 |

mations inasmuch as the general population is not stable because of the removal of individuals for experimental purposes. Moreover, it is difficult to classify specific deaths as active cases or carriers which died from some other cause. This applies particularly to the unweaned guinea pigs among which gross lesions may not be apparent. Hence, the specific deaths undoubtedly include a few cases which should be classed as carriers. It is believed, however, that the percentage mortality indicates roughly the progress of the infection. The population by months, the total deaths, the specific deaths and the per-

centage mortality for the period between July, 1925, and July, 1926, are given in Table I.

The data pertaining to the combined infections for the year beginning with July, 1926, when the second type first appeared, are given in Table II.

The percentage mortality for the two periods is presented in graphic form in Fig. 1.

The percentage mortality for the Type I infection displayed a considerable monthly fluctuation during the 2 endemic years beginning

TABLE II.

*Population, Total Deaths, Deaths from Paratyphoid and Percentage Mortality from Paratyphoid, July, 1926, through June, 1927.*

| Month | Population | Total deaths | Deaths from Paratyphoid Type I | Mortality Type I | Deaths from Paratyphoid Type II | Mortality Type II |
|---|---|---|---|---|---|---|
| | | | | *per cent* | | *per cent* |
| July | 571 | 14 | 7 | 1.22 | 1 | 0.17 |
| August | 468 | 48 | 30 | 6.41 | 3 | 0.64 |
| September | 382 | 32 | 17 | 4.45 | 3 | 0.78 |
| October | 463 | 11 | 0 | 0.00 | 5 | 1.07 |
| November | 432 | 13 | 2 | 0.46 | 5 | 1.15 |
| December | 417 | 64 | 4 | 0.95 | 22 | 5.27 |
| January | 436 | 40 | 3 | 0.68 | 13 | 2.98 |
| February | 424 | 35 | 1 | 0.23 | 11 | 2.59 |
| March | 406 | 49 | 1 | 0.24 | 24 | 5.91 |
| April | 454 | 57 | 5 | 1.10 | 18 | 3.96 |
| May | 407 | 42 | 1 | 0.24 | 11 | 2.70 |
| June | 449 | 35 | 0 | 0.00 | 5 | 1.11 |

July, 1925, and July, 1926, respectively. From the curve it may be seen that the highest rates, with a single exception, occurred during warm weather, namely: in July and September of 1925 and in February, June and Augusts of 1926. In general the infection showed a wave-like progress throughout the entire endemic stage. After the high rate of August, 1926, the percentage mortality declined and remained on a low level thereafter. The Type II infection appeared just prior to this peak rate of the initial type, in July, 1926. A relatively quiescent period of 5 months duration followed. During this time there was a slight but steady increase in the percentage mortality. The

period terminated in a sharp rise during December, 1926. There was a decline the 2 succeeding months with a second rise to a slightly higher level in March, 1927. Both of the peaks, it will be noticed, occurred during cold weather. The percentage mortality again declined and continued to fall through June at which time accurate observations were discontinued.

The breeding stock was the only group within the population which was sufficiently stable for a study of the cage spread of the Type II infection. The breeders are segregated in cages containing either four

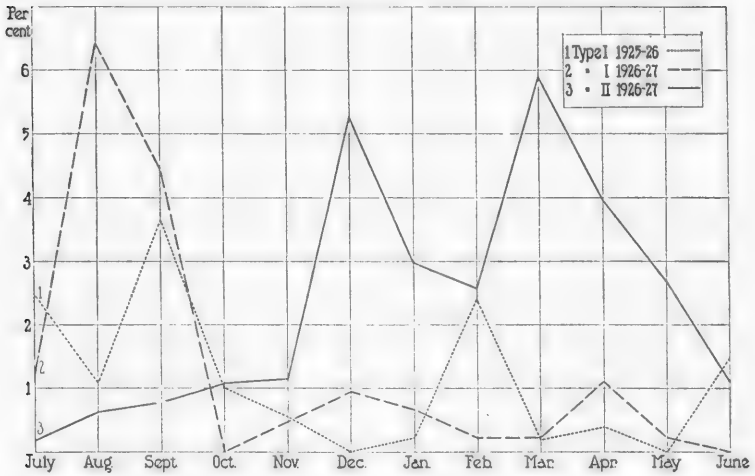



FIG. 1. Percentage mortality from Type I infection July, 1925, to June, 1927, and from Type II infection July, 1926, to June, 1927.

or five sows and one boar. Since the appearance of the second form of paratyphoid the sows have been removed to individual isolation units shortly before parturition. The young are born here and are left with their dams for a period of about 2 weeks when they are weaned. At this time the young are transferred to the stock, for general laboratory use, and the sows are returned to their original breeding cages. Until the suckling period is over the majority of young guinea pigs are subjected to direct contact only with their dams or within the litter. Occasionally a premature birth occurring in the

TABLE III.

*Spread of Type II Infection in the Breeding Cages July, 1926, to June, 1927.*

| | | | | | |
|---|---|---|---|---|---|
| 1. | 4. | 7. Dec. 26 C. | 10. Oct. 26 D. | 13. Aug. 26 C. | 16. |
| 2. Dec. 26 C.<br>May 27 C. | 5. Dec. 26 D.<br>Dec. 26 C.<br>Mar. 27 C.<br>Apr. 27 C. | 8. Dec. 26 D.<br>Feb. 27 D.<br>Mar. 27 C. | 11. | 14. Dec. 26 D.<br>Dec. 26 C.<br>Mar. 27 C. | 17. |
| 3. Jan. 27 D. | 6. Jan. 27 C.<br>Apr. 27 C. | 9. July 26 D.<br>Dec. 26 C.<br>Jan. 27 D.<br>Feb. 27 C.<br>Mar. 27 C.<br>Apr. 27 D.<br>Apr. 27 D.<br>June 27 D. | 12. | 15. May 27 C. | 18. Dec. 26 C. |
| 19. Oct. 26 D.<br>Feb. 27 D.<br>Mar. 27 C. | 25. Oct. 26 C.<br>Apr. 27 D.<br>Apr. 27 C. | 28. May 27 C.<br>May 27 D. | 32. Nov. 26 C. | 35. Mar. 27 C. | 39. Oct. 26 C.<br>Nov. 26 C.<br>Dec. 26 C.<br>Apr. 27 D. |
| 20. Sept. 26 C.<br>Jan. 27 D. | 26. Dec. 26 D.<br>Mar. 27 C.<br>Apr. 27 D. | 29. | 33. | 37. Feb. 27 C.<br>Mar. 27 D. | 40. Aug. 26 C.<br>Apr. 27 D. |
| | 27. Oct. 26 D.<br>Feb. 27 D.<br>Mar. 27 D.<br>May 27 C. | 31. Nov. 26 C.<br>Dec. 26 C.<br>Jan. 27 C.<br>Apr. 27 C. | 34. Jan. 27 C. | 38. Feb. 27 C. | 41. May 27 C. |
| 42. Sept. 26 C. | 44. June 27 C. | 49. Dec. 26 C.<br>Apr. 27 D. | 48. Feb. 27 D. | 51. | 54. |
| 43. | 46. | 52.<br>47. May 27 D. | 45. | 50. | 53. |

C., carrier; D., fatal case.

breeding cage brings them in contact with adult animals other than their dam.

Fatal cases of paratyphoid among the unweaned guinea pigs may be attributed in most cases to transfer of the organism from the dam. The transfer may be direct during intra-uterine life or indirect through the ingestion of contaminated feces or milk. Frequently the sow presents a normal appearance. These cases have been common with both types of infection. Formerly the sows were killed and autopsied. Throughout the second infection, however, most of them have been returned to the breeding cages and kept under observation. All of them were designated carriers. In addition, fatal cases have occurred from time to time among the sows, either in the breeding cages or in the isolation units. A cage record of the so called carriers and fatal cases has been kept since the onset of the second infection. A presentation of the data is given in Table III. Each square represents a cage in the order of its arrangement in the breeding room. The notations refer to individual sows. There are three sections of cages, as indicated.

Within a year after the first death from the second infection either fatal cases or carriers were detected in 32 out of 48 cages. The inmates of the remaining cages may have escaped infection entirely. It is more probable, however, that the repeated fecal examination of individuals would have revealed a small proportion of carriers. It is unlikely that there was any carriage of the infection from cage to cage except through the agency of the handlers.

The cages are of metal with solid tops and bottoms and are placed in rows on iron pipe racks. The rows are separated from each other by a wide air space. The cage arrangement precludes any possibility of direct leakage. In some instances infection may have been introduced through the addition of young sows to replace older ones removed by death or because of old age. These sows are drawn from the general stock which always contains a small number of carriers. With the exception of Cage 9, however, replacements have not been added to those cages with a high incid ıce of infection. It may be said that the breeding stock is cared for by two handlers. The feeding and cleaning in cages from 1 through 47 is always done by the same helper. Similarly, Cages 48 through 54 and in addition the isolation units are attended to by one man.

The spread of infection from cage to cage and within individual cages was gradual. The irregularity which is apparent with the cage

spread may be associated with the nature of the particular group. The breeding stock is composed largely of sows which normally breed and bear young four times a year. During pregnancy it is to be expected that the sow becomes more susceptible to bacterial invasion than during the postparturient periods. The ingestion of a normally tolerated dose of culture during the pregnant state might result in active disease. Or, in the absence of actual disease the lodgment of organisms in the uterus might result in a carrier state with subsequent infection of the young. Again, the ingestion of a sublethal dose of culture during a resting period with localization of the organism in the spleen, liver or intestinal tract might be followed by active disease during a subsequent pregnancy. It is suggested that the continual shifting of the sows together with periodic changes in the susceptibility of individuals were important factors in determining the cage spread of the organism.

In this connection it may be noted that no boars succumbed throughout the course of the second infection. It is to be expected that the number of fatal cases would be less than among the sows since the latter outnumber them five to one. The chances of the boars acquiring infection in the breeding cages are, however, somewhat greater since they remain there continuously except for a short interval during the summer. The sows, on the other hand, are periodically removed to isolation units while the young are suckling. It may be that the more vigorous nature of the boar is accompanied by a more active native resistance. It is believed, rather, that the weakening effect of successive pregnancies on the sow is accountable for the seeming difference in susceptibility. Among younger animals which have not been bred the incidence of fatal cases has shown no consistent difference.

## DISCUSSION.

Two years after the initial outbreak of paratyphoid the second infection appeared in the population. Following a quiescent period of 5 months the mortality from the latter type increased abruptly, declined slightly and again increased with a second decline. Both of these waves occurred during cold weather, the first in December, the second in March. Throughout this period the mortality resulting from the initial type was consistently low. The course of the second

infection was somewhat different from that of the initial one. The onset of the latter was abrupt, while that of the former was gradual. The mortality was greater with the initial disease and there was a corresponding increase in the number of fatal cases among adult animals. Apparently the population after long continued exposure to the first infection was better able to resist the second.

It seems clear, however, that the factors instrumental in holding the Type I infection to a low level were not equally active in combating the second type. A number of possible factors were discussed in a preceding paper (9). These will be reconsidered from the standpoint of their bearing on the spread of the second infection. It may be said that prior to the initial outbreak the guinea pig population had been free from specific disease, of epidemic proportions, for a period of 6 years. It was suggested that during this period there was an accumulation of individuals of low resistance. The natural removal of these animals during the early part of the epidemic left a stock better able to resist invasion and among which the infection ultimately declined to a low level. It may be supposed that natural selection continued to operate throughout the endemic stage tending to maintain a stock of normal resistance. It might be argued, however, that the less severe environmental conditions imposed upon the population during this long period would favor the reappearance of animals of low resistance. The unequal course of the two infections is, in part, opposed to such a view. Moreover, the reappearance of a weak strain of guinea pig should tend to increase the number of Type I cases. Actually these declined. It is suggested that the difference in the onset of the two infections may be attributed to the more uniform nature of the population, as regards natural resistance, which obtained at the appearance of the second type. The latter enjoyed a long free period which was not observed with the former.

With due regard to the significance of natural resistance and selection in checking the initial infection and delaying the second it is apparent that some additional factor or factors must have been operative. Fluctuation in the killing power and the invasiveness of the parasite are naturally suggested. As previously noted the question of a possible alteration in these characters with the Type I organism was not satisfactorily answered from experimental evidence (9). It was

tentatively suggested, however, that the organism was gradually adjusted to the population on a lowered level of virulence. Likewise, the possibility of similar differences between the second type and the initial one after its long sojourn in the guinea pig population has not been settled. Intraperitoneal injection of graded amounts of the two types into guinea pigs and mice failed to demonstrate any consistent variation. The inaccuracies inherent in the method employed are regarded as too great to warrant any final statement concerning the comparative invasive power of the two types.

Specific immunity was earlier considered as a factor in the decline of the initial infection. Its importance in the population at large was minimized but it was not denied that the resistance of certain groups might have been raised. It is certain that conditions were favorable for immunization within the breeding group during the endemic stage of the earlier infection. The Type I organism was widely disseminated throughout the cages of the unit and the ingestion of small doses of culture by individual sows must have commonly occurred. The postmortem examination of so called carriers, sows whose litters had shown one or more cases of paratyphoid, gave indirect evidence of an acquired immunity. In some instances healed or inactive lesions were found in the abdominal organs. The blood serum frequently agglutinated the homologous organism. It may be said, too, that a small number of sows examined shortly after the appearance of the second infection showed a much higher agglutinin titer against the initial organism than against the second type. There is a suggestion that natural immunization with the earlier form afforded a protection which was largely type-specific and accountable in part for the subsequent dominance of the second infection.

## SUMMARY.

The course is considered of a second type of Salmonella infection naturally appearing in a guinea pig population during the endemic stage of an earlier outbreak. After a quiescent period of 5 months the percentage mortality increased abruptly; fluctuated, with a second rise during the 9th month; and then declined. With the exception of a high rate during the 2nd month the percentage mortality from the initial infection tended to remain on a low level.

The spread of infection in the cages of the breeding stock is recorded from the time of the first fatal case. There was a slow but general dissemination of the second organism through the group. Fatal cases were confined solely to the sows. It is suggested that a lowered individual resistance occurring during pregnancy might be associated with the irregular cage spread and with the apparent difference in susceptibility of the sexes.

Natural host resistance, virulence of the organism and acquired host resistance are discussed from the standpoint of their bearing on the unequal distribution of deaths from the two infections.

## BIBLIOGRAPHY.

1. Nelson, J. B., *J. Exp. Med.*, 1927, xlvi, 541.
2. Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.
3. Pritchett, I. W., *J. Exp. Med.*, 1926, xliii, 143.
4. Topley, W. W. C., *J. Hyg.*, 1921, xix, 350.
5. Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353.
6. Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.
7. Sakai, K., *Tohoku J. Exp. Med.*, 1926, vii, 153.
8. Jordan, E. O., *J. Infect. Dis.*, 1925, xxxvi, 309.
9. Smith, T., and Nelson, J. B., *J. Exp. Med.*, 1927, xlv, 365.

# ETIOLOGY OF OROYA FEVER.

## X. Comparative Studies of Different Strains of Bartonella Bacilliformis, with Special Reference to the Relationship between the Clinical Types of Carrion's Disease and the Virulence of the Infecting Organism.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 7 to 10.

(Received for publication, November 5, 1927.)

Dr. Sebastian Lorente, Director of the National Department of Public Health of Peru, during a visit to New York in 1926, expressed the wish that experimental studies of Carrion's disease might be further extended and agreed to forward specimens of patients' blood to The Rockefeller Institute for the purpose. Six samples of blood were received from Dr. Lorente on December 29, 1926, and six more on April 1, 1927. Comparative studies of the strains of *Bartonella bacilliformis* isolated from these specimens form the subject matter of the present report. I wish at the outset to express to Dr. Lorente my appreciation of the cooperation which made the work possible.

### *Isolation of Bartonella bacilliformis from Specimens of Patients' Blood.*

The blood was collected in sterile vacuum bulbs containing dry sodium citrate sufficient to prevent coagulation (0.1 gm. per 10 cc. of blood). The containers were hermetically sealed and shipped in the steamer's refrigerator from Lima to New York, the time required for transportation being about 14 days. Three of the first six samples were unsuitable for cultural study, having become contaminated with extraneous bacteria by leakage of the container during transportation. The second shipment (Cases 7 to 12) arrived in perfect condition. *Bartonella bacilliformis* was obtained in pure culture from all six

specimens of the second lot and from the three uncontaminated samples of the first lot, nine strains in all being isolated.

The blood was inoculated into leptospira medium by the titration method, that is, several dilutions were made of each specimen (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000), and a tube of culture medium was inoculated for each dilution and one for the undiluted citrated blood, a total of six tubes of medium for each sample of blood. The amount of inoculum was 0.2 cc.

Dark-field examination revealed no motile organisms. In stained smears forms resembling *Bartonella* were found, but they were not sufficiently numerous or characteristic to be identified with certainty.

Case 1, F. A., 18 years old, resident of Canta, Department of Lima. Had been ill 3 months. Verruga suspected because of patient's residence and his symptoms. Erythrocytes 2,900,000. Leucocytes 7,000; neutrophils 72 per cent, eosinophils 1 per cent, monocytes 7 per cent, lymphocytes 20 per cent; no basophils. *Plasmodium falciparum* was present in the blood. Temperature 37.8°C. at time blood was taken.

Case 2, J. M., 45 years old, resident of Oroya, Department of Junin. Had been ill 2 months. Generalized miliary verrucous eruption as well as subcutaneous nodules. There had been fever of remittent type, but patient was afebrile at the time of taking blood. Erythrocytes 2,500,000. Leucocytes, 13,600; neutrophils 65 per cent, eosinophils 2 per cent, basophils 1 per cent, monocytes 10 per cent, lymphocytes 22 per cent.

Case 3, G. H., 20 years old, resident of Matucana, Department of Lima. Had been ill 15 days. Verrucous eruption of miliary type, scattered. Fever of remittent type still present, highest temperature 40°C. Erythrocytes 3,400,000. Leucocytes, 9,000; neutrophils 69 per cent, eosinophils 1 per cent, monocytes 8 per cent, lymphocytes 22 per cent; no basophils. Barton's bodies present, bacilliform type. Wassermann reaction positive.

Case 4, L. W., 37 years old, resident of Oroya, Department of Lima. Had been ill 1 month. Verrucous eruption of subcutaneous miliary form, scattered, localized chiefly in lower limbs. Patient had no fever at time blood was taken.

Case 5, A. F., 31 years old, resident of Chosica, Department of Lima. Had been ill 4 months. Miliary eruption and subcutaneous nodules were present on both arms and on thorax. Fever of remittent type still present; highest temperature 39.5°C., temperature at time blood was taken 37.8°C. Erythrocytes 1,920,000. Leucocytes 4,600; neutrophils 40 per cent, eosinophils 3 per cent, basophils 1 per cent, monocytes 9 per cent, lymphocytes 47 per cent. Wassermann reaction positive.

Case 6, F. T., 28 years old, resident of Matucana, Department of Lima. Had been ill 2 months. No eruption at time blood was taken, but for 1 month patient

had subcutaneous miliary lesions. Erythrocytes 2,460,000. Leucocytes, 6,400; neutrophils 73 per cent, eosinophils 2 per cent, monocytes 2 per cent, lymphocytes 23 per cent; no basophils.

Case 7, S. N., 22 years old, resident of Puruhuay (Chosica), Department of Lima. No fever. Generalized verrucuous eruption of miliary and nodular types. Erythrocytes 3,480,000. Leucocytes 4,400; neutrophils 72 per cent, eosinophils 1 per cent, basophils 1 per cent, monocytes 4 per cent, lymphocytes 22 per cent.

Case 8, J. A., 33 years old, resident of Canta, Department of Lima. Had been ill 6 months. No fever. Generalized miliary eruption. Erythrocytes 3,100,000. Leucocytes, 4,000; neutrophils 65 per cent, eosinophils 1 per cent, monocytes 8 per cent, lymphocytes 26 per cent; no basophils.

Case 9, J. M., 19 years old, resident of Chosica, Department of Lima. Had been ill 15 days. Continuous fever, 39°C. No eruption. (Malignant verruga?) Erythrocytes 1,250,000; normoblasts 4 per cent, erythroblasts 2 per cent. Anisocytosis, anisochromia, polychromatophilia. Leucocytes 8,400; neutrophils 73 per cent, eosinophils 2 per cent, monocytes 3 per cent, lymphocytes 22 per cent; no basophils. Barton's bodies present, bacilliform type predominating. Jolly bodies also present.

Case 10, E. C., 39 years old, resident of Chosica, Department of Lima. Had been ill 1 month. No fever. Verrucous eruption, miliary and nodular, scattered. Erythrocytes, 5,080,000. Leucocytes 5,200; neutrophils 44 per cent, eosinophils 7 per cent, basophils 1 per cent, monocytes 7 per cent, lymphocytes 41 per cent. Barton's bodies of coccoid type present.

Case 11, M. C., 27 years old, resident of Chosica, Department of Lima. Had been ill 12 days. No fever, and no eruption at time blood was taken. Erythrocytes 1,080,000; normoblasts 9 per cent, erythroblasts 2 per cent, neutrophilic myelocytes 3 per cent. Megalocytes. Leucocytes 22,200; neutrophils 88 per cent, monocytes 3 per cent, lymphocytes 9 per cent. No eosinophils or basophils. Barton's bodies, coccoid form, present.

Case 12, M. L., 28 years old, resident of Matucana, Department of Lima. Had been ill 1 month. No fever. Eruption of miliary type, discrete. Erythrocytes 4,800,000. Leucocytes 4,900; neutrophils 54 per cent, eosinophils 5 per cent, monocytes 10 per cent, lymphocytes 31 per cent; no basophils. Barton's bodies present, coccoid form predominating.

## *Comparative Studies of the Strains Isolated.*

### *Appearance of Growth (30°C.).*

On leptospira medium there is a light grayish, usually homogeneous, but sometimes finely granular, translucent layer of growth at the top of the column of medium. Single isolated colonies form denser central masses and are surrounded by a hazy zone of outgrowth from the periphery. All the strains pre-

sented a similar appearance except Nos. 9 and 12, which grew faster and more densely than the others. Horse blood agar plates or slants yield minute grayish, raised, shiny, firm miliary colonies which appear transparent but are grayish when covered with a layer of saline solution. They adhere to the medium and are difficult to scrape from the surface. Strains 9 and 12 form somewhat larger colonies, which reach a diameter of 2 mm. within 4 or 5 days.

### *Motility (Dark-Field Observations).*

Cultures on leptospira medium or horse blood agar slants or plates contain very actively motile forms for the first 4 to 6 days, especially in the condensation water of the blood slants, but the organisms invariably become motionless sooner or later. The flagella apparently become abnormally large under the unfavorable conditions existing in a semisolid medium and are thrown off; for detached flagella, so large as to be visible by dark-field examination, appear in cultures about a week old (Figs. 32 and 33).

I have been unable to detect any differences in movement among the strains studied; they all exhibit rotation and progression, singly or in groups of two or three, or in masses. Occasionally there is no motility at all even in young cultures, owing undoubtedly to unfavorable conditions of cultivation.

### *Staining Properties and Dimensions.*

All the strains are frankly Gram-negative and take basic fuchsin rather poorly. Giemsa's solution gives satisfactory results and brings out the fairly sharp contours of the organisms in cultures which have been grown 4 to 6 days on the surface of blood agar slants or plates. This definition of the form is quickly lost as the cultures grow older. Organisms grown on leptospira medium or in the condensation water of a blood agar slant stain only poorly, and it is often impossible to recognize individuals; nevertheless they thrive well and remain viable under these conditions much longer than on the surface of slants or plates. There is a tendency for most of the strains to take up the stain more intensely in the interior portion of the body than on the outer layer. Often the chromatophilic material is seen to be heaped up toward either end, giving the appearance of a diploid coccobacillus. Sometimes the accumulation is in nodular masses at irregular intervals throughout the entire length, the effect simulating a chain of ill stained cocci, while in shorter forms there may be only a single deeply stained dot. The organisms often assume a kind of triangular or elongated wedge shape.

In fresh preparations of young, actively motile cultures, the organisms all appear to be of the same size and shape, but certain variations in size and form are recognizable in stained smears (Plates 7 and 8). Special care has been taken to exclude various external factors which might influence the comparison. For example, cultures have been grown on the same medium (horse blood agar plates prepared on the same day with the same material) for 6 days at 30°C.

under identical conditions, and film preparations have been made in the same way (the culture put into a drop of distilled water, the mixture spread on the slide, and dried in the air for a given period), fixed in methyl alcohol for 5 minutes, and then stained with Giemsa's solution in the same jar for 30 minutes. Another set of slides has been treated by Gram's method and counterstained with dilute carbol fuchsin, washed, blotted, and dried in the air. The measurements given in Table VI, therefore, should be fairly reliable. Strain 7 (Figs. 5 and 6) is distinctly coarser and Strain 11 (Figs. 13 and 14) decidedly finer than the rest. There is, however, a gradual transition, some approaching the coarser type and others the finer, hence striking differences are brought out only by comparing the two extremes. Some strains are short, others more rod-shaped.

### *Flagella (Zettnow-Fontana Combination Stain).*

Determination of the type of flagella in newly isolated pathogenic microorganisms is not of morphological interest merely; it may prove to be of immunological importance. Orcutt[1] has demonstrated that the serological specificity of certain organisms resides in the flagella, and Bauer[2] recently found in this laboratory that serological types of tetanus bacilli possess dissimilar flagella; for example, Type III, having a single unipolar heavy spiral flagellum, and Type IV, having peritrichal, rather coarse regularly wavy flagella, of moderate length, are quite distinct from the other types. All strains of *Bartonella bacilliformis* were therefore stained for flagella by a modified procedure, in which Zettnow's mordant is followed by reduction with Fontana's ammoniac silver nitrate. The cultures used were those utilized for the measurements of size, that is, the surface growth on horse blood agar plates after 6 days at 30°C.

Certain strains proved to have comparatively shorter, smoother, and more delicate flagella than others (Plate 9). Strains 3, 9, 10, 11, and 12 (Figs. 22, 26 to 29) are of this type, while Strains 2, 7, 8 (Figs. 21, 24, and 25), and the two old strains, S.A.[3] and P.5[4] (Fig. 30) have distinctly more wavy or spiral and coarse appendages. The number of flagella seems to vary among different strains, some showing a single flagellum, others as many as four. In all instances, however, the flagella are unipolar. Short flagella scarcely exceed 3$\mu$, long ones may measure 10$\mu$ or more.

### *Pathogenicity.*

*Macacus rhesus* monkeys were used to determine the virulence of the original samples of blood, as well as of the newly isolated cultures,

[1] Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 43, 627.
[2] Bauer, J. H., not yet published.
[3] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.
[4] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

the tests being made by three different methods. (1) The effects of local inoculation of the original samples of blood were simultaneously compared by intradermal inoculation of each specimen into separate sites on the abdominal skin of the same monkey (the experiment was done in duplicate), previous experience having shown[5] that an experimental local lesion in *Macacus rhesus* remains confined to the site of inoculation, and that several intradermal inoculations with different materials may be made simultaneously on the same animal. (2) The effect of local inoculation of each of the cultures isolated from the blood

TABLE I.

*Tests of Infectivity of Blood, Cases 1 to 6. Multiple Intradermal Inoculations, Dec. 29, 1926.*

| Case No. | *M. rhesus* 1A | *M. rhesus* 2A |
|---|---|---|
| 1 | — | — |
| 2 | — | — |
| 3 | — | — |
| 4 | — | — |
| 5 | — | — |
| 6 | — | — |
| Blood cultures, Jan. 6, 1927. | | |
| | — | — |
| Blood cultures, Jan. 24, 1927. | | |
| | + (1:100) | + (1:100) |

was compared in the same animal with that of the inoculation of passage strains of known virulence. (3) Each strain was individually tested on a monkey by both intradermal and intravenous inoculation.

The first two types of test have an advantage over the third in that the variations in the susceptibility of individual monkeys is not involved. The third method is the more usual one, but in testing a large number of strains it is necessary to economize in monkeys, hence the factor of individual variation may enter into consideration. The results in this series of animals, however, were determined not only by local reactions but also by blood cultures at appropriate intervals.

[5] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 455.

For virulence tests, cultures grown for 8 days on leptospira medium at 30°C. and those grown for 4 to 6 days on horse blood agar slants and plates at the same temperature were pooled, the mixture comprising first, second, and third generation cultures of a given strain. The original samples of blood were tested again simultaneously with the cultures, a procedure which yeilded interesting results, inasmuch as the blood, in contrast to the cultures, produced no cutaneous lesions. The results of the various tests are outlined in the tables.

Table I summarizes the results of the direct pathogenicity tests of the blood of Cases 1 to 6 inclusive. No local lesions were induced,

TABLE II.

*Parallel Infectivity Tests of Blood of Cases 2, 3, and 5, and of Cultures Obtained from the Blood. Multiple Intradermal Inoculations, Feb. 7, 1927.*

| Case No. | | *M. rhesus* 3A |
|---|---|---|
| 2 | Blood | − |
| | Culture | ++ |
| 3 | Blood | − |
| | Culture | ++++ |
| 5 | Blood | − |
| | Culture | ++++ |

though invasion of the blood by *Bartonella bacilliformis* occurred, as shown by the results of blood culture in both animals 26 days after inoculation. The cultures from Cases 1, 4, and 6 were contaminated, those from Cases 2, 3, and 5 yielded marked local lesions (Table II; Fig. 31). Similar results were obtained with the specimens from Cases 7 to 12, the original blood being non-virulent for the skin of the monkey, while the cultures in three instances (Cases 9, 10, 11) gave rise to typical verruga lesions. Parallel tests of the blood and the cultures in Cases 7 to 12 are recorded in Tables III and IV. Strains 7 to 12 were tested separately on different monkeys (Table V) with results which agree with those of the multiple tests.

The experiments show that of nine strains tested, six possessed definite specific pathogenicity for *Macacus rhesus;* the other three

were non-pathogenic, as shown by three separate tests. The three non-virulent strains (7, 8, and 12) came from cases of benign miliary or nodular verruga, in two of which the microscopic examination of the blood had been negative for *Bartonella;* in Case 12 *Bartonella* was present in the blood, though the red count was practically normal. The six virulent strains, with the exception of Strain 10, came from patients whose blood showed either microscopically detectable num-

TABLE III.

*Parallel Infectivity Tests of Blood of Cases 7 to 12 and of Cultures Obtained from the Blood. Multiple Intradermal Inoculations, May 3, 1927.*

| Case No. | | *M. rhesus* 4A | *M. rhesus* 5A |
|---|---|---|---|
| 7 | Blood | – | – |
| | Culture | – | – |
| 8 | Blood | – | – |
| | Culture | – | – |
| 9 | Blood | – | – |
| | Culture | +++ | +++ |
| 10 | Blood | – | – |
| | Culture | +++ | +++ |
| 11 | Blood | – | – |
| | Culture | ++++ | ++++ |
| 12 | Blood | – | – |
| | Culture | – | – |
| Control (P. 5 strain, from verruga nodule) | | ++++ | ++++ |

bers of *Bartonella bacilliformis* or marked anemia, or both. In three of these cases there were no skin lesions, and the red counts were very low (1,080,000 to 1,250,000). These represent the pure septicemic form of Carrion's disease, without cutaneous involvement.

The fact that the virulent strains came from the severe types of Carrion's disease, and the non-virulent ones from benign verruga seems to indicate that the highly fatal disease is caused by strains possessing a greater virulence. The first strain of *Bartonella bacilli-*

*formis* isolated,[3] which was obtained from the blood of a fatal case of Carrion's disease, was found to produce in monkeys of average susceptibility the clinical picture of a human case of verruga, but in unusually susceptible animals, which are rather rare, it induced a fatal infection similar to that of human Oroya fever. The strains from the severe

TABLE IV.

*Parallel Infectivity Tests of the Original Blood, of Cultures on Leptospira Medium, and of Blood Broth Mixtures, Cases 7 to 12. Multiple Intradermal Inoculations, May 18, 1927.*

| Case No. | *Macacus rhesus* 6A | | | |
|---|---|---|---|---|
| | Original blood | Cultures | | |
| | | Leptospira medium | Blood broth | |
| | | | 30° | 37° |
| 7 | − | − | − | − |
| 8 | − | − | − | − |
| 9 | − | +++ | − | − |
| 10 | − | ++ | − | − |
| 11 | − | + | − | − |
| 12 | − | + | − | − |

TABLE V.

*Individual Tests of Strains 7 to 12. Intradermal and Intravenous (2 cc.) Inoculations, July 1, 1927.*

| Strain No. | Monkey No. | Skin lesions | Blood cultures |
|---|---|---|---|
| 7 | 7A | − | − (Aug. 8, 1927) |
| 8 | 8A | − | − |
| 9 | 9A | + | + (1:10) |
| 10 | 10A | +++ | + (1:100,000) |
| 11 | 11A | ++++ | − |
| 12 | 12A | − | − |

cases of the present series have not so far, in the small number of monkeys inoculated, reproduced the fatal Oroya fever, but they have invariably induced cutaneous lesions similar to those occurring in benign human cases, while the strains isolated from the blood of benign verruga have not proven sufficiently virulent to set up any infection in the same monkeys.

TABLE VI.

*Summary of Data.*

| *B. bacilliformis* strain No. | Clinical data | | | | | Observations on cultures | | | | | | | | Pathogenicity for *Macacus rhesus* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Measurements | | | Flagella | | |
| | Fever | Erythrocyte count | *Bartonella* in blood | Eruption | Duration of illness | Cultural titer of blood | Growth on leptospira medium | Growth on blood agar | Predominant forms | Extremes | | Character | Length | |
| | | | | | | | | | | Width | Length | | | |
| | | | | | | | *30°C.* | *30°C.* | | | | | | |
| 2 | | 2,500,000 | | General miliary | 2 mos. | 1:10,000 | Light grayish haze | Minute, almost transparent, raised, shining, firm, round colonies | 0.3 × 0.8μ | 0.25–0.35μ | 0.5 –1μ | Unipolar, 2 or more, smooth | 3–5μ | + |
| 3 | | 3,400,000 | + | Few eruptions | 15 days | 1:1,000 | " | " | 0.3 × 1μ | 0.25–0.4μ | 0.4 –1.2μ | Similar, but spiral | 3–5μ | + |
| 5 | | 1,920,000 | | Miliary and nodular | 4 mos. | 1:10,000 | " | " | | | | | | + |
| 7 | – | 3,480,000 | | General miliary and nodular | | 1:10 | " | " | 0.4 × 1.4μ | 0.3 –0.4μ | 0.8 –1.6μ | Unipolar, 1 or 2; spiral | 5–10μ | – |
| 8 | – | 3,100,000 | | General miliary | 6 mos. | 1:10 | " | " | 0.3 × 1.2μ | 0.25–0.3μ | 0.5 –1.4μ | Same as 7 | 5–10μ | – |
| 9 | 39° | 1,250,000 | + | – | 15 days | 1:10,000 | " (denser) | " (coarser) | 0.3 × 1μ | 0.25–0.3μ | 0.4 –1.4μ | Unipolar, 2 or 3; smooth or wavy | 3–6μ | + |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | − | 5,080,000 | + | Few miliary nodular | 1 mo. | 1:1,000 | Light grayish haze | Minute, almost transparent, raised, shining, firm, round colonies | 0.35 × 1μ | 0.3 –0.4μ | 0.3 –1.2μ | Unipolar, 1 to 3; smooth or slightly wavy | 3–8μ | + |
| 11 | − | 1,080,000 | + | − | 12 days | 1:100,000 | " | " | 0.2 × 1μ | 0.2 –0.25μ | 0.3 –1.2μ | Same as 10 | 3–8μ | + |
| 12 | − | 4,800,000 | + | Few miliary | 1 mo. | 1:100 (denser) | " | " (coarser) | 0.3 × 0.7μ | 0.3 –0.4μ | 0.5 –1.2μ | " " 10 | 3–5μ | − |
| S.A. | | | | | | | Light grayish haze | Minute, almost transparent, raised, shining, firm, round colonies | 0.25 × 1μ | 0.25–0.35μ | 0.45–1μ | Unipolar, 2 to 4; spiral | 5–10μ | + |
| P. 5 (nodule) | | | | | | | " | " | 0.25 × 1μ | 0.2 –0.3μ | 0.3 –1.2μ | Same as S.A. | 5–10μ | + |

As has previously been suggested, the variety of types of human *Bartonella* infection may be accounted for by variations in the susceptibility of different individuals or different tissues of the same individual, or by variations in the virulence of the microorganism, or by both. The present experiments appear to emphasize more particularly the factor of variation in virulence of the parasite. According to clinical observations, the skin lesions more usually follow the acute febrile anemic stage (*fiebre grave de Carrion*, or Oroya fever); the lesions in these instances are evidently caused by a strain virulent enough to have invaded the blood. Yet there are cases in which the infection is chiefly confined to the cutaneous tissues, the anemia being very mild, and strains isolated from such cases would presumably be less virulent, as was found to be true in the present experiments. In instances in which the severe bartonellemia supervenes in the course of an apparently benign verruga, we may conclude that the strain belonged to the virulent type, but that the individual was of the resistant type, and that the defenses of the resistant organism were finally overcome by continued constitutional disturbances, due perhaps to the presence of the parasite in question, perhaps to some other cause. Phenomena of this sort are not uncommon in infections with other organisms which may give rise to septicemia following local infection (streptococcus, staphylococcus, the tubercle bacillus).

### *Serologic Studies.*

Suspensions of *Bartonella bacilliformis* which would be suitable for agglutination tests are difficult to obtain, owing to the fact that the masses of organisms are well nigh impossible to disperse. Complement fixation tests proved easier to interpret.

Two samples of immune sera, obtained by inoculating rabbits intravenously on several occasions at appropriate intervals with live cultures of Strain S. A.[3] were employed against a non-fixing dose of saline suspensions of plate cultures (horse blood agar) of each strain of *Bartonella bacilliformis*. The suspensions were heated at 60°C. to kill the microorganisms and the immune sera inactivated at 56°C.

All the strains gave complete fixation with the antiserum with the exception of Strain 12, which gave partial fixation (30 per cent). The

results indicate that on the whole the strains belong to the same serologic group.

## DISCUSSION.

Carrion's disease offered a singularly complex problem, because of its two clinically dissimilar aspects, the grave fever of Carrion (Oroya fever), and benign verruga. The presence of the endoglobular bodies discovered by Barton[6] practically settled the etiology of the severe cases, but the relation of Barton's bodies to the cutaneous lesions of verruga remained obscure, since it had not been proven that the same bodies were present in the blood or skin lesions of patients with the mild cutaneous disease. Moreover, while the cutaneous tissues affected were definitely infective, inducing similar skin lesions in monkeys, the parasites present in such large numbers in the blood of patients with malignant anemia, with or without skin lesions, appeared to be non-infective for monkeys, inducing neither skin lesions nor systemic infection so far as could be determined. It has recently been shown, however, that the apparently negative findings were not due to the absence of *Bartonella bacilliformis*, but to the fact that, except in severe systemic infection, the parasite cannot be detected by microscopic examination, its presence being revealed only by a suitable culture method.[7] The unsolved portion of the problem of the etiology of Carrion's disease has been cleared up by the cultural procedure, which permits the ready isolation of *Bartonella bacilliformis* and its detection even when it is present in extremely small numbers.

The importance of the selection of the culture method cannot be overemphasized, since on the special features of the medium used depended the success of the entire investigation. Had blood agar been used for the isolation experiments—and it is by far the best of the many other media tried—many strains would undoubtedly have been missed, some because growth of the microorganism is not uniformly successful on this medium, some because of loss of virulence due to the frequent subculturing required with blood agar cultures. Once the parasite had been obtained in culture on the semisolid leptospira medium, and its special properties studied, it became possible to discover the causes

[6] Barton, A. L., *Crón. med.*, Lima, 1909, xxvi, 7.
[7] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

for the apparently conflicting clinical and experimental evidence. Moreover, by special histological technique[8] it was shown that *Bartonella bacilliformis* is microscopically detectable in large numbers in human and experimental lesions, where it had previously escaped detection.

The reason for the difference in pathogenicity between the endoglobular parasites, which are present in such large numbers in verruga maligna, and those existing in the skin lesions of verruga benigna has not been brought to light. The fact that in the first mentioned condition the microorganism is incapable of inducing in monkeys anything more than a slight transitory blood invasion, detectable only by blood culture, explains the negative results obtained by the Harvard Commission[9] with blood from severe Oroya fever, results which seemed unmistakably to indicate a totally different etiology for Oroya fever and verruga peruana.

SUMMARY.

Through the cooperation of Dr. Sebastian Lorente, Director of the National Department of Public Health of Peru, nine strains of *Bartonella bacilliformis* have been isolated, by means of the semisolid leptospira medium, from nine of twelve specimens of blood withdrawn from cases of verruga and forwarded from Peru under conditions of refrigeration. The cultural titer of the blood specimens immediately after their arrival (2 weeks after withdrawal) varied from 1:10 to 1:100,000. Blood from the severe anemic type of the disease, in which there was no eruption, had the highest titer. Blood agar slants yielded irregular results, but some strains grew well on these media.

[8] The technique is that used by Nicholson (*J. Exp. Med.*, 1923, xxxvii, 221) for the detection of *Rickettsia* in tissues. Fixation in Regaud's fluid (*Arch. Anat. Micr.*, 1910, xi, 296), which consists of 4 parts of 3 per cent potassium bichromate and 1 part of commercial formalin, is followed by staining with Giemsa's solution, according to the method recommended by Wolbach (*J. Med. Research*, 1919–20, xli, 1). 1.25 cc. of Giemsa's solution (Grübler's, for bacteria) is diluted with 1.5 cc. of methyl alcohol and 50 cc. of distilled water to which has been added 3 drops of 1 per cent sodium bicarbonate.

[9] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburu, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

Morphologically the strains differed very little in fresh preparations examined by dark-ground illumination. In stained preparations some strains appeared coarser, others finer than the average. Special staining indicated that the flagella were characteristically unipolar and varied in number from one to four, some strains showing distinctly more wavy and heavier flagella than others. Young cultures grown on the surface of horse blood agar for 3 to 6 days show individuals with fairly sharp contours, short rods, often varying in thickness toward one or both ends, being intermingled with smaller oval or coccoid elements. Some strains show a predominance of bacillary, some of coccobacillary forms. It is not known whether these features are inherent or are due to conditions of growth, which, though identical, may react differently upon different strains. Definiteness in outline disappears with the age of the culture.

More striking variations are found in the virulence of the different strains for the monkey (*Macacus rhesus*). Three of the nine strains isolated proved to be non-pathogenic for the monkeys. All three of these were derived from cases of benign verruga. The remaining six strains all gave rise to local lesions when intradermally inoculated and were recovered in culture from the blood of the animals. So far, severe anemia has not developed in any of the monkeys.

It is significant that most of the severe cases yielded virulent strains, while some of the strains from benign verruga were non-pathogenic. It appears highly probable that the severe form of Carrion's disease is, in general, caused by a virulent strain, while the benign forms are due to a strain of low virulence. On the other hand, a virulent strain may cause benign verruga in unusually resistant persons and a weak strain may give rise to severe blood infection in unduly susceptible individuals. The form of Carrion's disease is probably determined primarily by the inherent virulence of the strain of *Bartonella bacilliformis* and is modified secondarily by individual predisposition in a given case.

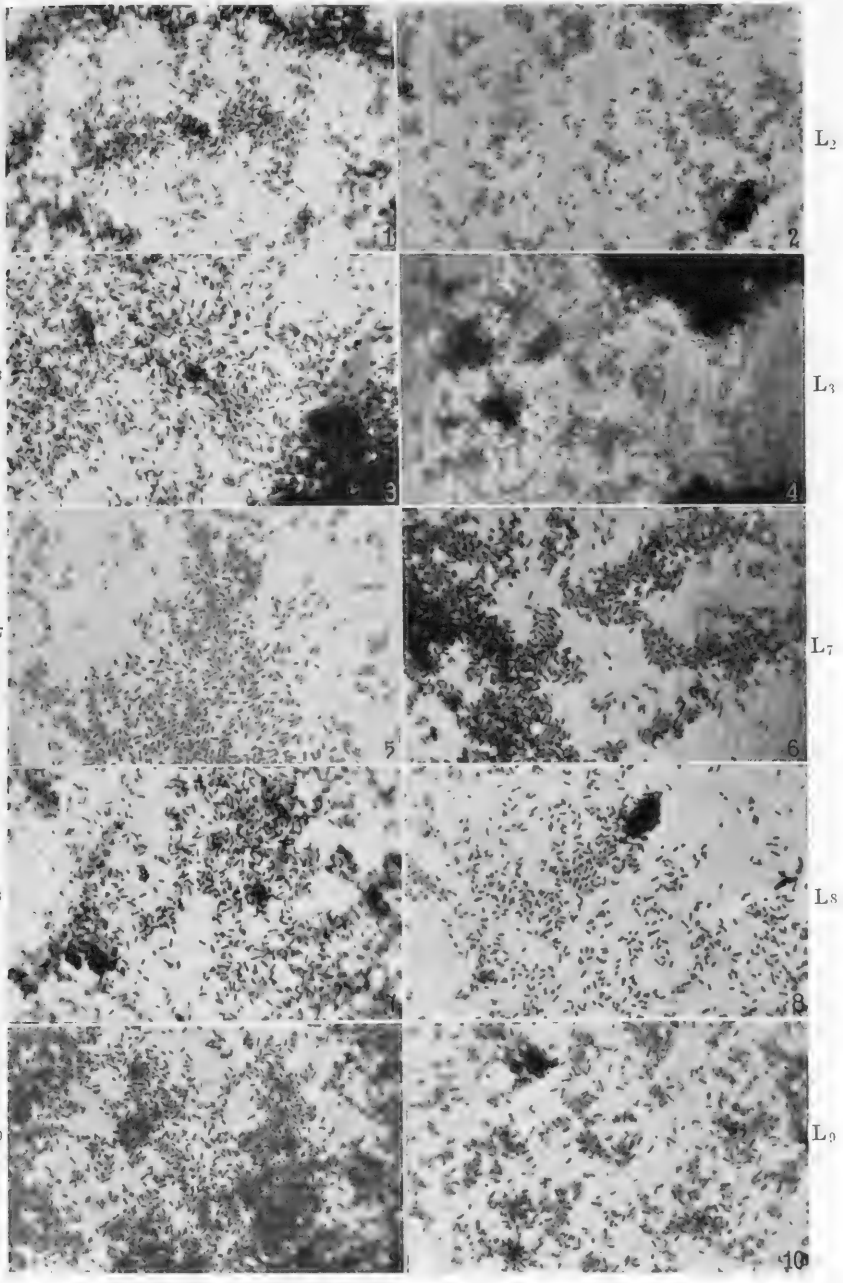
An interesting phenomenon brought out by the present investigation was the failure of the nine human blood specimens to induce local verruga in the same monkeys in which the corresponding cultures, inoculated simultaneously at separate sites, gave rise to typical lesions. Yet the original blood samples were shown by cultivation to have

contained live bartonellas at the time they were inoculated, and blood culture revealed the presence of the microorganisms in the blood of monkeys which showed no other signs of infection after inoculation with the human blood. Whether this striking difference is merely a quantitative one or is due to some factor still unknown—such as, for example, a biological phase of the microorganism—has not been determined. The uniformly negative results of transmission experiments with blood by previous investigators is explained by an actual inability of the blood to induce skin lesions and the lack, until now, of a reliable method of detecting *Bartonella bacilliformis* in the monkeys' blood.

The strains isolated showed similar serologic properties, as tested by complement fixation.

Comparison of different strains of *Bartonella bacilliformis*. × 1000.

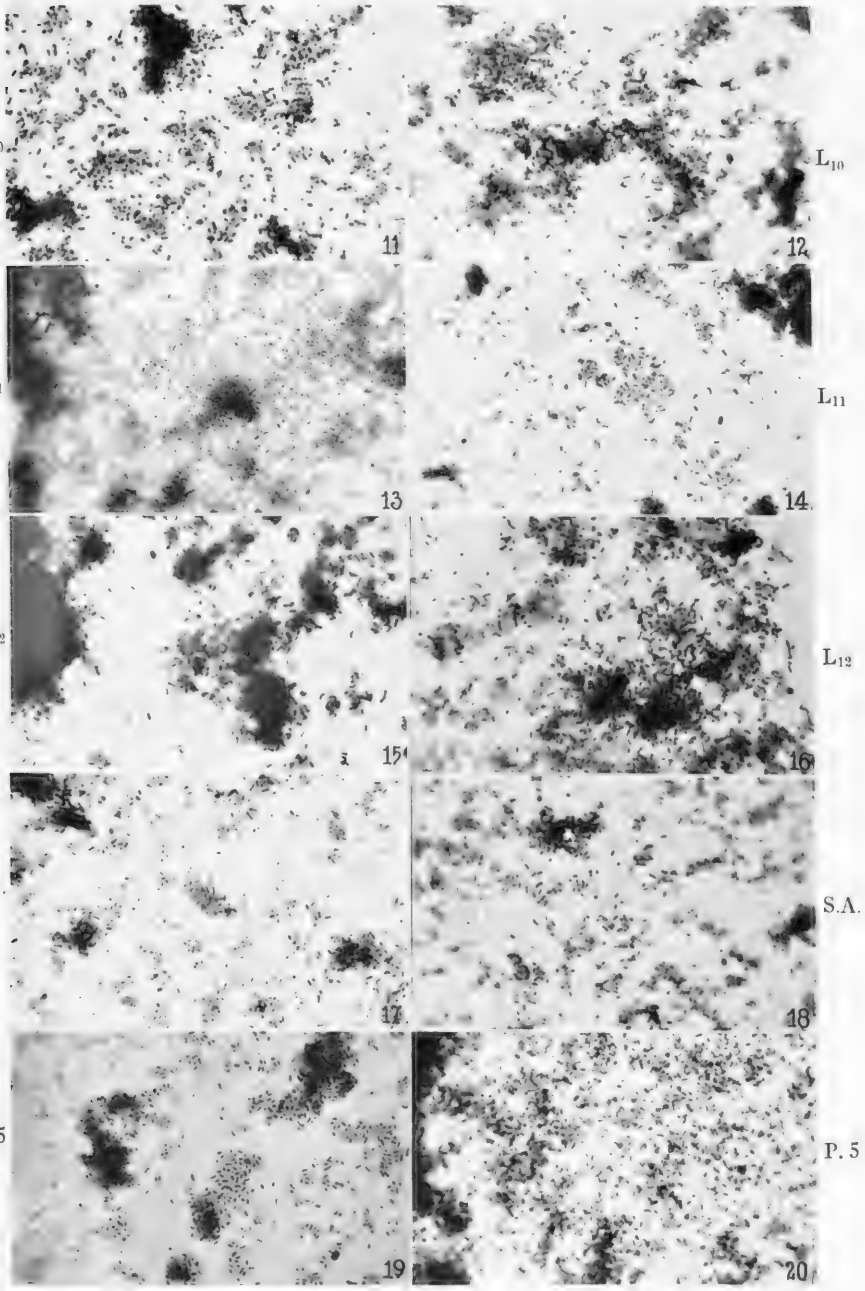
Giemsa's stain. Gram's stain, counterstained.




(Noguchi: Etiology of Oroya fever. X.)

Comparison of different strains of *Bartonella bacilliformis*. × 1000.

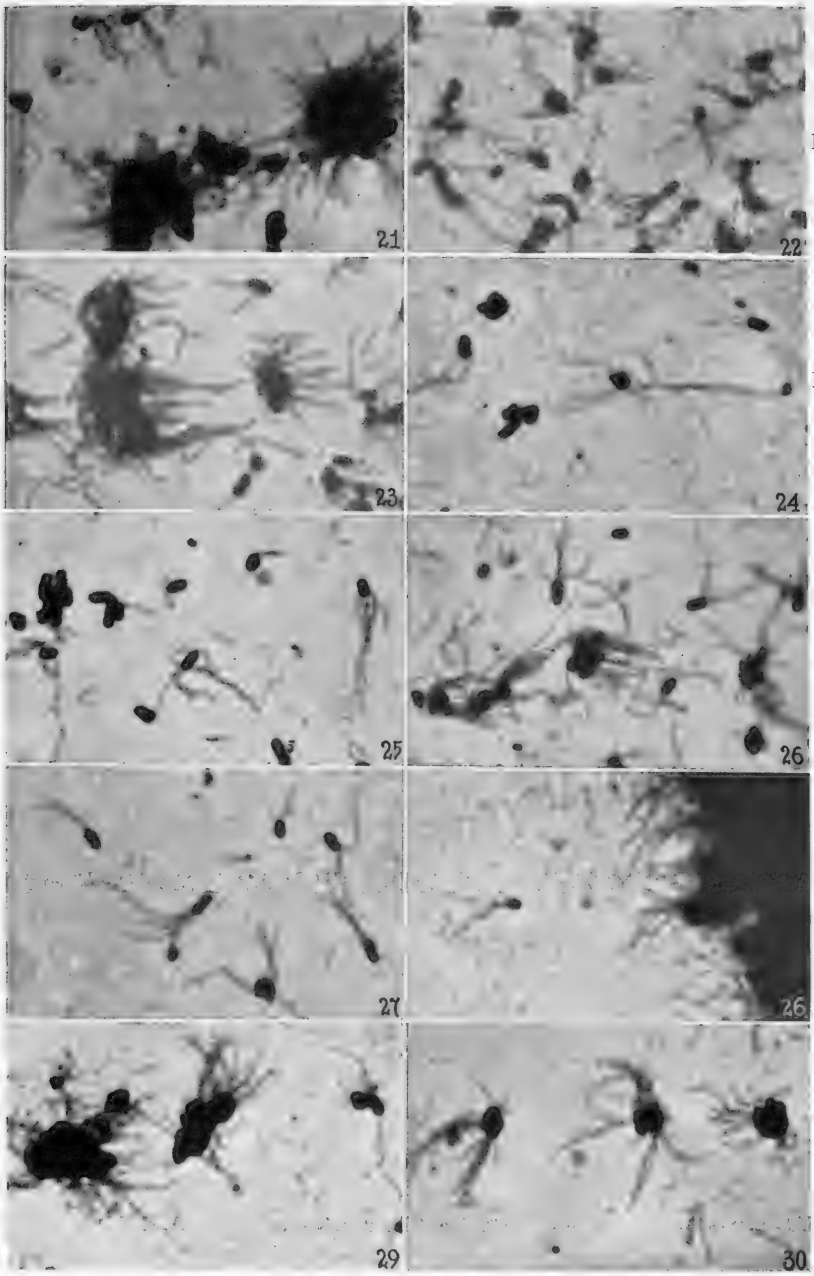
Giemsa's stain. Gram's stain, counterstained.



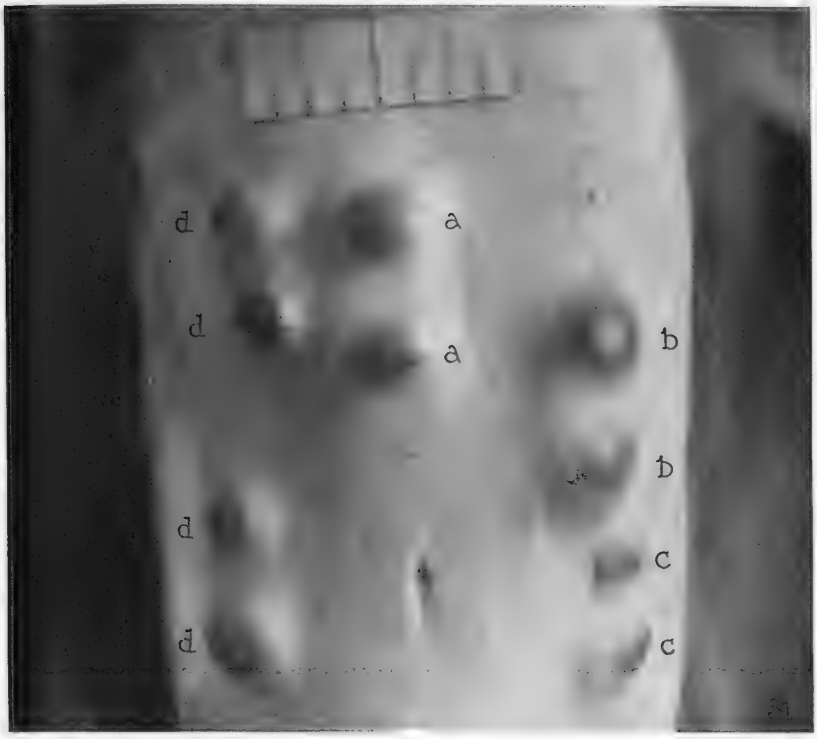

(Noguchi: Etiology of Oroya fever. X.)

Comparison of different strains of *Bartonella bacilliformis*. × 2000.

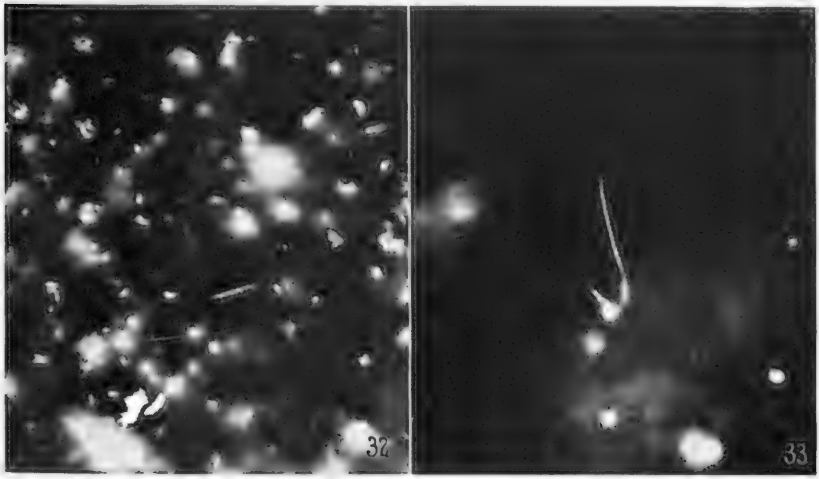
Zettnow-Fontana stain.




(Noguchi: Etiology of Oroya fever. X.)




Lesions produced by cultures of (*a*) Strain 2, (*b*) Strain 3, (*c*) Strain 5, and (*d*) Strain P. 5. 30 days after inoculation. *M. rhesus* 3A. Natural size.




Abnormal detached flagella. Dark field. × 1000.

UNIV. OF MICH.

# ETIOLOGY OF OROYA FEVER.

## XI. Comparison of Bartonella Bacilliformis and Bartonella muris. Cultivation of Bacterium murium, N. Sp.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 11 and 12.

(Received for publication, November 14, 1927.)

Mayer,[1] in 1921, discovered, in the red blood cells of a rat which was suffering from severe anemia as a result of trypanosomiasis and chemotherapy, peculiar inclusion bodies, resembling *Bartonella bacilliformis* of Oroya fever, which he designated *Bartonella muris*. Subsequently Lauda[2] reported that splenectomy of white rats brought about a severe anemia which could be transferred to normal rats by inoculating them with the blood or a suspension of liver tissue of the splenectomized animals. The name "infectious anemia of the rat" was introduced to describe the condition. Mayer and his collaborators[3] suspected that the anemia resulting from extirpation of the spleen might be due to *Bartonella muris* and succeeded in demonstrating the inclusion bodies in splenectomized rats. Their assumption was that *Bartonella muris* is present in most rats, and that the invasion of the blood is made possible by the removal of a defense organ, the spleen.

Because of the close analogy between *Bartonella muris* and *Bartonella bacilliformis*—their morphological similarity, their localization in the erythrocytes, and their association with a severe form of anemia, a comparative study has been made of these two bartonellas, the specific subjects of inquiry being: (1) the percentage incidence of appearance of *Bartonella muris* in the blood of splenectomized rats and mice, (2) the result of splenectomy in animals other than rats

[1] Mayer, M., *Arch. Schiffs- u. Tropenhyg.*, 1921, xxv, 150.

[2] Lauda, E., *Arch. ges. Physiol.*, 1925, cclviii, 529.

[3] Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Woch.*, 1926, v, 559.

and mice, (3) the transmissibility of *Bartonella muris* to normal rats, (4) the transmissibility of *Bartonella muris* to other animals, especially splenectomized animals, (5) the susceptibility to *Bartonella bacilliformis* of rats and other animals after splenectomy, (6) the comparative morphological features of *Bartonella muris*, *Bartonella bacilliformis*, and *Grahamella talpæ*, the latter an intracorpuscular parasite of moles described by Graham-Smith,[4] Thomson,[5] and Brumpt,[6] and (7) the cultivation of *Bartonella muris*.

Splenectomy was performed on fifty white rats, three wild rats, three Chinese hamsters (*Cricetulus griseus*),[7] and nine white mice. *Bartonella muris* appeared after splenectomy in the blood of all, in some numerously, in others sparsely. Splenectomy always gives rise to an increase in the number of blood platelets, some of which showed very much more distinct chromatin rods and granules than the platelets of normal animals. These loosely packed or partially liberated thrombocyte bodies are sometimes very easily confused with *Bartonella muris*, especially when lying over or under the erythrocytes.

Splenectomy was carried out also in eight *Macacus rhesus*, two Java monkeys, four rabbits, and four guinea pigs. No intracorpuscular elements resembling *Bartonella muris* or *Bartonella bacilliformis* appeared in the blood of any of these animals, nor did the animals show any anemia comparable to that observed in the splenectomized rats or mice.

### *Transmissibility of Bartonella muris.*

Repeated attempts have been made to reproduce the anemic condition and cause the appearance of *Bartonella muris* in the blood of normal rats and mice by injection of blood or suspensions of liver tissue (Lauda's method) from splenectomized animals, intravenously, intraperitoneally, intradermally, or intratesticularly. Such experi-

[4] Graham-Smith, G. S., *J. Hyg.*, 1905, v, 453.

[5] Thomson, J. D., *J. Hyg.*, 1906, vi, 574.

[6] Brumpt, E., *Bull. Soc. Path. Exot.*, 1911, iv, 514.

[7] I am indebted for these animals (which were obtained primarily for other experiments) to Dr. Carl TenBroeck, who procured them for me in Peking, and to Dr. Amos Wong, who was so kind as to bring them to the United States in his personal care.

ments have always been negative in this respect. Nor has it been possible to transmit the condition to splenectomized monkeys, rabbits, or guinea pigs.

In some instances, however, a quite different condition was induced.

In Rats 54 and 55, inoculated intratesticularly with a suspension of the liver of Rat 40, which had shown a marked blood invasion by *Bartonella muris*, the testicles became acutely inflamed. Rat 54 was castrated on the 6th day of disease. The condition persisted in Rat 55 for 10 days, but by the 14th day the testicle had healed. *Bartonella muris* was not found in the blood of either of these animals. Histological study of the excised testicle of Rat 54 showed acute infiltration of polymorphonuclear leucocytes into the acini, together with edema, congestion, and focal necrosis. Rather coarse bacilli were present in the lesion, some having been taken up by the leucocytes. From the testicular tissue was obtained a pure culture of a microorganism to be described later (Strain 54).

Two normal rats, 56 and 57, inoculated intraperitoneally and intratesticularly with the suspension of testicular tissue of Rat 54, became very ill within 48 hours, and in the blood of Rat 56, taken on the 3rd and 4th days, were found a few intracorpuscular bodies suggestive of *Bartonella muris*. The animal died before the 5th day. Rat 57 showed similar elements in the erythrocytes on the 3rd day but none thereafter. It died of diarrhea in 10 days. There was only slight induration of the testicles in these two animals.

A similar result was obtained in one of three normal rats inoculated with citrated blood of splenectomized Rat 41 (*B. muris* ++). In this case (Rat 66) a bilateral intratesticular inoculation had been made. The inflammation of the testicles reached its maximum on the 5th day and receded during the 3 following days. The same microorganism as had been isolated from Rat 54 was obtained from the aspirated fluid (Strain 66). In the other two animals (Rats 64 and 65), in which the inoculation was unilateral, there was no induration. Examination of blood films of these three animals failed to reveal the presence of *B. muris*. Three other normal animals inoculated intratesticularly and intraperitoneally with a suspension of the liver of Rat 41 showed neither testicular induration nor invasion of the blood by *B. muris*.

There is apparently present in the blood or liver of some splenectomized rats a microorganism capable of setting up acute orchitis in normal rats. The transient appearance of intracorpuscular bodies in the two rats inoculated with the testicular tissue of Rat 54 was far from convincing evidence of transmission of *B. muris*. The failure of most of the attempts to transmit the organism shows at least that regular transmission is not readily accomplished in American rats.

Mayer,[8] in Hamburg, encountered a similar difficulty. It would, indeed, seem unusual that a microorganism which is unable to invade the blood until the spleen is removed should acquire the power to infect the blood of a normal animal by transfer from the splenectomized one.

It has been found that the intraperitoneal deposition of a small fragment of normal rat or mouse spleen, or the intraperitoneal injection of a saline suspension of the tissue, into splenectomized rats or mice will cause the rapid disappearance of *Bartonella muris* from the blood. Hence *B. muris* is extremely sensitive to splenic substances, even when the spleen itself is not present.

*Susceptibility to Bartonella bacilliformis of Splenectomized Animals.*

Experiments to be reported elsewhere have shown that the spleen has no important defensive function against the invasion of *Bartonella bacilliformis*. While the blood culture titers of splenectomized monkeys infected with *Bartonella bacilliformis* were usually higher than those of control animals of the same series, yet variations in blood titer are so common in monkeys infected with this microorganism that they cannot be taken as significant in this instance. Rabbits, guinea pigs, and rats were not any less resistant to infection with *Bartonella bacilliformis* after splenectomy than are these animals normally.[9]

*Comparative Morphology of B. muris, B. bacilliformis, and Grahamella talpæ in the Blood* (*Plate 11*).

Comparison of blood films stained with Giemsa's solution shows *B. muris* (Fig. 2) to be smaller than *B. bacilliformis* (Fig. 1). There is also less variation in form in *B. muris*, which usually appears as short rods or coccoid forms whose size varies within a very small range. *B. bacilliformis*, on the other hand, though often having an oval or coccoid form similar to that of *B. muris*, frequently occurs in long, slender rods, and *Y* or *V* forms are very common (Fig. 1). The long slender forms usually have square ends, as though the or-

[8] Mayer, M., Borchardt, W., and Kikuth, W., *Arch. Schiffs- u. Tropenhyg.*, 1927, xxxi, 295.

[9] Noguchi, H., *J. Exp. Med.*, 1927, xliii, 851.

ganism had been cut transversely. Shorter rods may be bent at a sharp angle at one or two places, showing that they are composed of two or three individuals. These branching forms are not found in preparations of *B. muris*, which resembles *Grahamella talpæ* more than it does *B. bacilliformis*, though differing from *Grahamella* in its manner of distribution. I am indebted to Dr. A. C. Coles, of Bournemouth, England, for Giemsa-stained preparations of the blood of two English moles showing *Grahamella* infection (Fig. 3). The grahamellas are often found in such numbers in a single cell as to fill the stroma completely; *B. muris* is usually scattered among many cells, and many individuals are found outside the cells. The preparations show two types of *Grahamella*, one distinctly and uniformly thick, as compared with *B. muris*, and suggesting a bacterium, the other more delicate and showing beaded formations. No branched forms or long threads have been seen in the films of *Grahamella*.

*B. muris*, *B. bacilliformis*, and *G. talpæ* have similar staining reactions with Giemsa's solution, but basic fuchsin, while giving sharp definition of *B. bacilliformis*, fails to stain *B. muris*.

*B. muris* of the rat is indistinguishable from the similar elements which appear in the blood of wild rats or Chinese hamsters after splenectomy. The forms found in the corpuscles of splenectomized mice are more granular and show beaded formations.

### *Cultivation of B. muris.*

The etiologic rôle of *B. muris* in the anemia of splenectomized rats is difficult to establish because of the fact that no animal has so far been found which is susceptible to the organism under natural conditions, *i.e.*, when the spleen is present. Once the spleen is removed, *B. muris* appears in the blood, hence splenectomized animals cannot be used for demonstration of the pathogenic effects of a microorganism which has been isolated.

In the course of repeated attempts at cultivation, I have isolated two different organisms, both of which resemble *B. muris* in morphology. The first (Strain 28 A, Figs. 7 and 8) came from the blood of a splenectomized rat and grew on leptospira medium as a grayish layer at the surface. It did not grow on ordinary culture media. It was

subsequently found that this organism had the features of a minute diphtheroid. It had no pathogenicity whatever for normal rats.

From the blood of the same animal (Rat 28) another minute, apparently non-motile, Gram-negative bacterium was isolated on a blood agar plate (Strain 28 B, Figs. 4 to 6). This organism grows on blood agar in very minute colonies but not on leptospira medium or on ordinary culture media. On a blood agar slant it grows more readily, and after 3 or 4 days at 30° or 37°C. the dew-like discrete surface colonies gradually spread to a diameter of 3 mm. or more and, when the surface is densely seeded, coalesce to a shiny, faintly bluish gray, moist layer of growth. The condensation water becomes slightly turbid. This organism is not an acid-producer like the first, but is hemolytic. Morphologically, especially when stained for a short time (20 minutes) with Giemsa's solution it appears very much like *B. muris*. The most interesting feature of the organism is its ability to set up an acute orchitis in normal rats when intratesticularly injected.

The first experiment was made on Rat 42, into which a 24 hour culture grown on blood slants at 30° and 37°C. was injected intraperitoneally and intratesticularly. *B. muris* did not appear in the blood, but the testicle became indurated within 6 days, when the animal was killed for transfer. A suspension of the testicular tissue was used for cultivation experiments and for intraperitoneal and intratesticular inoculation of two normal rats, 52 and 53. A pure culture of the organism was isolated from the testicular tissue. No induration followed the intratesticular injection of the tissue suspension, but in the blood of Rat 52 a few forms resembling *Bartonella muris* were detected 8 days after the inoculation.

The virulence tests of Strain 28 B were repeated on two normal rats, 58 and 59, the cultures being injected intratesticularly only. An acute orchitis developed in both animals, and they died 10 and 9 days after inoculation, respectively. *B. muris* was not found in the blood at any time. From the testicles of Rat 59 pure cultures of the organism were recovered.

Pure cultures of Strain 28 B from Rat 42 were tested on two normal rats, 60 and 61, the injections being made directly into the testicles. Rat 60 reacted more vigorously than Rat 61, the inflammation of the testicle reaching its maximum in 3 days and disappearing in 9 days, while Rat 61 showed only a slight induration on the 3rd day. Bodies resembling *B. muris* were found in very small numbers in the blood of Rat 61, but not in Rat 60. A saline suspension of the testicular tissue of Rat 42, kept at 4°C. for 7 days, was injected into two normal animals, 62 and 63, but in neither was there more than a very slight induration of the testicles. *B. muris* was not found in the blood.

Strain 28 B proved, therefore, to be identical in pathogenicity and cultural properties with the strains isolated from Rats 54 and 66. All three pathogenic strains are evidently the same microorganism. The culture forms are small, measuring 0.4 to 0.8 $\mu$ in length and 0.2 to 0.3 $\mu$ in width, and appear very much like *Bartonella muris*. The organisms found in the indurated testicular tissues were coarser

TABLE I.

*Summary of Experiments with the Organism Cultivated.*

| Source of culture | Strain No. | Result of inoculation |
|---|---|---|
| Blood of splenectomized Rat 28 | 28 A | Non-pathogenic |
| Blood of splenectomized Rat 28 | 28 B | Induced testicular lesion in Rat 42. Culture recovered from testicular tissue<br>Suspension of testicular tissue induced slight induration of testicles in Rats 62 and 63 |
| Testicular tissue of Rat 42, inoculated with culture of Strain 28 B | 28 B | No testicular lesions in Rats 52 and 53. Few intracorpuscular elements suggestive of *B. muris* seen in blood of Rat 52<br>Induration of testicles in Rats 58, 59, 60, 61. Culture recovered from Rat 59 |
| Testicular tissue of Rat 54, inoculated with suspension of liver tissue of splenectomized Rat 40 | 54 | Slight induration of testicles in Rats 56 and 57. Intracorpuscular elements in small numbers in blood of both |
| Testicular punctate of Rat 66, inoculated with citrated blood of splenectomized Rat 41 | 66 | Not tested |

(1 to 1.5 $\mu$ by 0.3 to 0.4 $\mu$), took a deeper stain, and showed very little resemblance to the delicate *B. muris* of the blood of splenectomized rats.

Several normal rats, rabbits, and guinea pigs were injected intratesticularly with pooled cultures of Strains 28 B, 54, and 66 (from Rats 28, 42, 54, 59, and 66). The rats showed the usual marked induration of the testicles, but the other animals reacted only slightly. *B. muris* did not appear in the blood of any of the animals.

*Immunization Experiments.*

Several normal rats were inoculated intraperitoneally on several occasions with live cultures of Strains 28 B, 65, and 66, and 9 to 10 days after the last injection the animals were splenectomized. Following the splenectomy *B. muris* promptly appeared in the blood of all, that is, previous active immunization with the microorganism in question conferred no protection against the invasion of *B. muris* on subsequent splenectomy.

An immune serum pepared in a rabbit by repeated intravenous injections with live cultures of different strains of the pathogenic microorganism failed to cause the disappearance of *B. muris* from the blood of splenectomized rats or to prevent invasion of the blood by *B. muris* thereafter when given intraperitoneally immediately before splenectomy.

Although neither active nor passive immunity could be induced in rats against *B. muris*, by means of the microorganism under study, this organism is not definitely proven not to be *B. muris* since according to recent observations of Mayer and his coworkers no immunity develops in splenectomized rats as a result of previous infection, the organisms appearing again in the blood of recovered individuals when blood containing them is intracardially injected. In the case of *B. bacilliformis* killed cultures do not constitute a potent vaccine, and an immune serum prepared in rabbits by repeated intravenous injections of live cultures of the organism confers little or no protection against a virulent strain of the parasite, the incidence of takes among vaccinated and control monkeys being practically the same.

The identity of the pathogenic microorganism cultivated from splenectomized rats cannot be established until suitable experimental animals are found. In the meantime I propose for it the provisional name, *Bacterium murium*.

*Bacterium murium* was not isolated from the blood of 20 normal rats. Its invasion of the blood of splenectomized animals was perhaps due to the removal of the spleen.

SUMMARY.

*Bartonella muris* appeared in the blood of all white rats, wild rats, Chinese hamsters, and mice, from which the spleen was removed, but

did not appear in that of splenectomized monkeys, rabbits, or guinea pigs.

It has not been possible to transmit *B. muris* to normal rats, monkeys, rabbits, or guinea pigs, by intraperitoneal, intradermal, or intravenous injection of blood containing *B. muris* from splenectomized rats.

In two instances an acute orchitis was induced in normal rats by injection directly into the testicle of blood or saline suspensions of the liver of splenectomized rats. The intracorpuscular elements occasionally found in the blood of some of the animals could not be definitely identified as *B. muris* or as having appeared as a result of the inoculation. The acute orchitis of rats was transferable to normal rats in series.

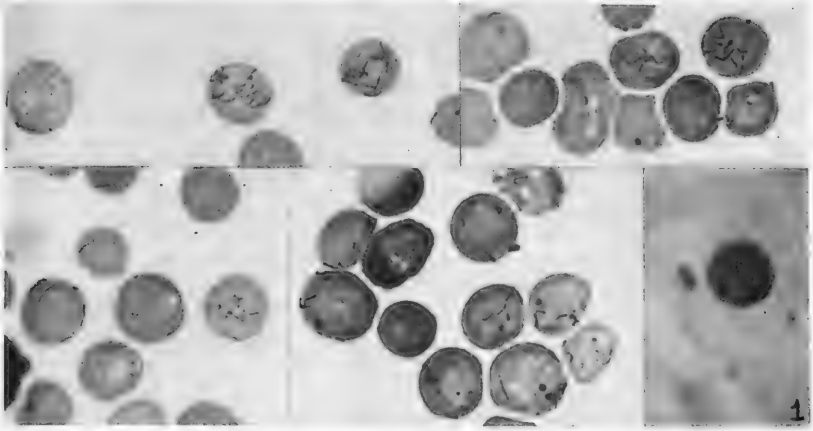
From the testicular tissue, as well as directly from the blood of a splenectomized rat, there was isolated in pure culture a microorganism which induced in the testicles of normal rats an acute orchitis such as resulted from inoculation of the blood or liver suspensions of splenectomized rats. While a few inclusions were found in the erythrocytes of some of the animals, their number was so small and their occurrence so infrequent that they could not be definitely identified as *B. muris*.

In morphological features the cultural forms of the microorganism isolated resemble *B. muris*. The organism found in the testicular tissues, however, is considerably coarser than *B. muris* and takes a deeper stain.

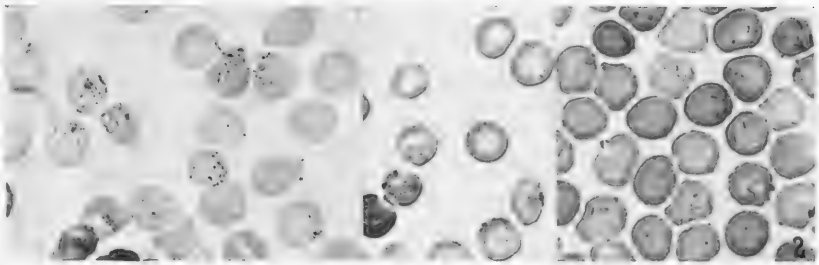
Immunological study failed to settle the question of the relation between *B. muris* and the cultivated organism, which is provisionally called *Bacterium murium*.

*Bartonella muris*, *Bartonella bacilliformis*, and *Grahamella talpæ* have characteristic individual morphological features.

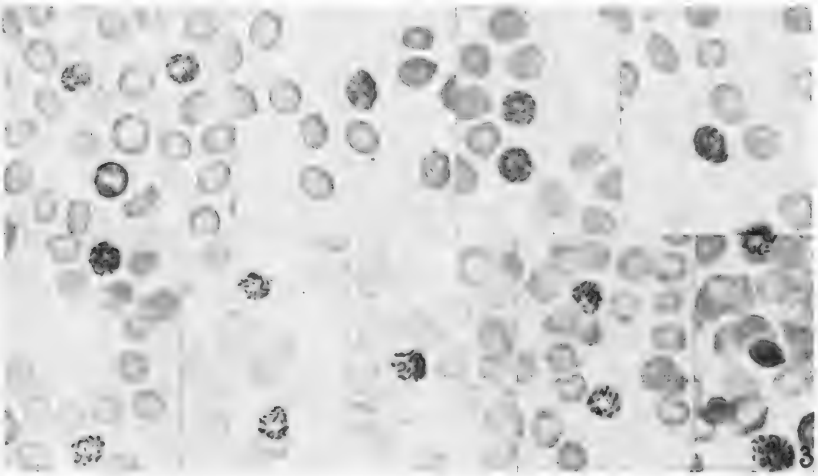
Giemsa's stain. × 1000.



*Bartonella bacilliformis* in human blood.



*Bartonella muris* in blood of splenectomized rats.



*Grahamella talpæ* in blood of English moles, Coles Strains 1 (above) and 2 (below).

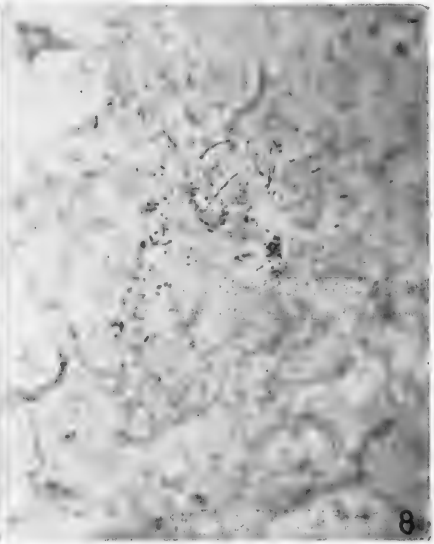
Cultures from blood of splenectomized rats.

Strain 28B. Blood agar. 48 hrs. Natural size.

Strain 28B. Blood slant. 24 hrs. Dark field. × 1000.

Strain 28B. Blood slant. 48 hrs. Giemsa's stain. × 1000.

Strain 28A. Dark field. × 1000.



Strain 28A. Giemsa's stain. × 1000.

# THE EFFECT ON SUBSEQUENT AGGLUTINATION OF THE EXPOSURE OF BACTERIA TO HEATED ANTISERUM.

By F. S. JONES, V.M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

(Received for publication, November 9, 1927.)

Eisenberg and Volk (1) have shown that bacteria treated for intervals of 1 to 2 hours with immune serum heated to 65°C. or 70°C. failed to agglutinate when the heated serum was removed and fresh immune serum added. Ehrlich's conception is that agglutinin is composed of two substances, one that brings about the combination of cell and antibody and another that produces agglutination. His explanation of Eisenberg and Volk's phenomenon assumes that the zymophore substance is changed by heat into agglutinoid which no longer produces clumping. On the other hand, the haptophore portion is unaltered and still combines with the cell to such an extent that further union cannot take place on the addition of unaltered immune serum. This has been accepted as the explanation of the phenomenon.

It seemed possible that Eisenberg and Volk's phenomenon might be explained in other ways. Any serum when heated and added to a bacterial suspension might stick to the surface of the bacterial cell and thus prevent specific union on the subsequent addition of unheated immune serum. Furthermore, it is necessary to show by experiment that the agglutinin or antibody has been completely destroyed when the serum is heated in order to explain the process on the basis of agglutinoid combination. This is especially true in the light of the observations of Beyer and Reagh (2), Orcutt (3), and F. S. Jones (4) that certain types of agglutinin are not destroyed when heated at 70°C. for 20 minutes. A further suggestion is that when too concentrated heated serum is added the reaction may not take place because

of an excess of protein. To answer the questions thus brought up a series of experiments was undertaken.

## EXPERIMENTAL.

The agglutinin was prepared by the immunization of rabbits with a motile strain of the hog cholera bacillus and *Bacillus abortus*. The agglutinating serum had been stored in the refrigerator for a month or more. Details of procedure are recorded under the separate experiments.

*Experiment 1.*—The growth from two 24 hour agar slants of the motile hog cholera bacillus was suspended in 2 cc. NaCl solution. Amounts of 0.3 cc. of this suspension were added to 1.5 cc. of a 1:1 dilution of hog cholera bacillus antiserum which had been heated at 75°C. for 30 minutes; to normal rabbit serum diluted 1:1 and also heated at 75°C.; to normal rabbit serum diluted 1:1; and to 2 cc. of cow serum heated at 65°C. for ½ hour. For control purposes the same amount of culture suspension was added to 1.5 cc. of NaCl solution. All tubes were incubated 1½ hours, then 8 cc. of sterile salt solution was added and the tubes centrifuged rapidly. The supernatant liquid was withdrawn and the bacilli resuspended in 10 cc. of salt solution. The suspensions were then tested with hog cholera bacillus serum. Readings were made after 2 hours incubation and refrigeration overnight.

This experiment was repeated with similar results. From the protocol submitted it is evident that exposure to normal serum fails to appreciably affect the agglutinability of the bacilli. However, when the culture was added to the specific serum which had first been heated, the addition of further agglutinin did not materially increase the subsequent agglutination. It is well known from the work of others that 75°C. does not completely destroy the flagellar agglutinin, and as indicated in the control tube, some agglutination had already taken place. During the exposure the contents of each tube were repeatedly agitated by drawing up and rapidly expelling the mixture with capillary pipettes, so that any clumps were readily broken. In addition, after centrifugation the bacilli in the first series were difficult to resuspend and the suspension was relatively unstable since deposition occurred throughout the tubes of the series. It is possible that a little agglutinin still remaining in the serum combines with the bacterial cells, but through mechanical means the clumps may be largely broken so that the bacterial suspension no longer reacts markedly with its antiserum.

It will be noted in the first experiment that heating agglutinin to 75°C. confirmed in a large measure the findings of Eisenberg and Volk. Their experiments were conducted after heating the agglutinin to 65°C. or 70°C. When the lower temperatures were tried with the hog cholera bacillus agglutinin the bacilli were promptly agglutinated, and although the suspensions were agitated vigorously the bacilli settled to the bottom of the tubes after resuspension in salt solution.

It seemed of further interest to observe the effect on subsequent

TABLE I.

*The Effect on Subsequent Agglutination of Exposing the Hog Cholera Bacillus to Various Sera.*

| | Dilutions of unheated hog cholera bacillus immune serum | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | Control |
| Bacilli exposed to hog cholera bacillus antiserum diluted 1:1 and heated at 75°C. for ½ hr. | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + |
| Bacilli exposed to heated rabbit serum | C | C | C | C | C | +++ | + | − | − | − |
| Bacilli exposed to unheated rabbit serum | C | C | C | C | ++ | ++ | − | − | − | − |
| Bacilli exposed to heated cow serum | C | C | C | C | C | +++ | + | ± | − | − |
| Bacilli exposed to NaCl solution | C | C | C | C | C | +++ | ++ | + | ± | − |

Agglutination has been reported as follows: C, the maximum, with heavy deposit and complete clearing; ++++, not quite complete; +++, strong clumping; ++, well defined clumping and a definite deposit in the bottom of the tube; ±, a slight deposit.

agglutination of exposure of the bacilli to immune serum heated at higher temperatures. Experiment 2 covers this phase of the question.

*Experiment 2.*—The immune and normal rabbit sera were each diluted in 4 parts of salt solution. A portion of each was heated at 75°C. for 20 minutes, another lot was exposed to 80°C. for the same period. To each 2.5 cc. of the heated diluted serum, 0.3 cc. of a heavy suspension of living hog cholera bacilli was added. The same amount of culture was added to NaCl solution for control purposes. All tubes were incubated 1 hour and the contents mixed repeatedly with capillary pipettes. After incubation, 8 cc. of NaCl was added and the mixture centrifuged. The supernatant was then poured off and the bacilli resuspended in 10 cc. of salt

solution. The suspensions were then tested with immune serum. The findings are given in Table II.

It will be noted that the data submitted in the second experiment confirm the first observation. When the bacilli are first submitted to antiserum which has been heated at 75°C. for 30 minutes, they fail to agglutinate to any great extent on the addition of fresh agglutinin. When the immune serum is heated at 80°C. for 30 minutes, the effect is less marked although the agglutinin titer of the unheated serum is appreciably diminished. The effect cannot be ascribed to mechanical

TABLE II.

*The Effect of Exposing the Hog Cholera Bacillus to Serum Heated at 75°C. and 80°C.*

| | Dilutions of hog cholera bacillus immune serum | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | Control |
| Bacilli exposed to hog cholera bacillus antiserum diluted 1:4 and heated at 75°C. for ½ hr. | ++ | + | ± | ± | ± | ± | − | − | − | − |
| Bacilli exposed to hog cholera bacillus serum diluted 1:4 and heated at 80°C. for ½ hr. | C | C | C | ++++ | +++ | ++ | − | − | − | − |
| Bacilli exposed to normal rabbit serum diluted 1:4 and heated for ½ hr. at | | | | | | | | | | |
| 75°C. | C | C | C | C | C | ++++ | + | ± | − | − |
| 80°C. | C | C | C | C | C | ++++ | + | − | − | − |
| Bacilli exposed to NaCl for the same period | C | C | C | C | C | ++++ | + | − | − | − |

influences of serum or slowing down of motility, since the organisms exposed to heated rabbit serum or salt solution continued to agglutinate well with the immune serum. Data not given in the table show that immune serum heated at 90°C. for 30 minutes has no appreciable influence on subsequent agglutination.

It seemed of further interest to ascertain to what extent the proteins of the immune and normal rabbit sera actually combined with the bacterial cells. The writer (5) had previously shown that collodion particles and in certain cases bacteria bathed in solutions of various proteins retained sufficient on their surfaces so that they were agglu-

tinated on the addition of precipitin specific for the sensitizing protein. With a similar procedure it was hoped that actual combination could be shown.

*Experiment 3.*—As in the previous experiment, the immune and normal rabbit sera were diluted 1:4. A portion of each was heated at 75°C. and 80°C. for ½ hour. To 3 cc. portions of diluted serum 0.3 cc. of bacterial suspension was added. They were then incubated 1 hour, an excess of salt solution was added, and the tubes centrifuged. The bacilli were then washed twice more in salt solution and finally resuspended in 10 cc. NaCl. The resuspensions were tested with anti-rabbit serum precipitin prepared by injecting a fowl with rabbit serum. The results are given in Table III.

TABLE III.

*The Effect of Rabbit Serum Precipitin on Hog Cholera Bacilli First Exposed to Heated Sera.*

| | Dilutions of rabbit serum precipitin | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:50 | 1:100 | 1:200 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | Control |
| Culture first treated with antiserum heated at 75°C. for 20 min. | ++++ | ++++ | +++ | ++ | + | + | + | ± |
| Culture first treated with antiserum heated at 80°C. for 20 min. | ++++ | ++ | + | ± | ± | − | − | − |
| Culture first treated with normal rabbit serum heated at 75°C. for 20 min. | ++ | ± | − | − | − | − | − | − |
| Culture first treated with normal rabbit serum heated at 80°C. for 20 min. | ++ | ± | − | − | − | − | − | − |
| Culture carried in NaCl solution | ± | − | − | − | − | − | − | − |

From the protocol submitted in Table III, it is evident that when immune serum containing antibody has been heated at 75°C. for 20 minutes, certain of the antibodies are still capable of combining with the bacterial cell and such combination may be detected by precipitin. Thus during union of bacillus and antibody certain of the serum proteins are deposited on the bacteria, and this protein deposit will react with its specific antibody and agglutination result. That there had been considerable deposition of serum protein on the bacilli exposed to the serum heated at 75°C. is clear. It is also true that serum heated at 80°C. is not as efficient in this regard, but nevertheless considerable

must be deposited on the bacterial cell surfaces. The reaction in this instance is well defined, since agglutination occurs when as little as 1/200 cc. of precipitin is added. The control series in which the bacilli were acted upon by normal serum show only slight fixation between bacterium and rabbit serum protein. The bacilli carried in salt solution, but otherwise manipulated in a similar manner, failed to agglutinate when treated with the rabbit serum precipitin.

As a further confirmation of the preceding experiments the following experiment may be briefly cited.

*Experiment 4.*—When 0.5 cc. of bacillary suspension is added to a suitable amount of complement and 0.5 cc. of antiserum diluted 1:4, and, after suitable incubation, amboceptor and red cells are added, no hemolysis results. The same holds true when the diluted antisera are heated for 20 minutes at 70°C. and 75°C. When the antiserum has been heated to 80°C. there is still considerable diversion of complement (a ++ reaction). When the antiserum is heated to 90°C. even less of the complement is diverted although the hemolysis is not complete.

As the experiment shows, there remains in the serum heated at 70°, 75°, and 80°C. an antibody still capable of combining with its antigen in sufficient quantities to entirely or partially divert complement.

When another organism and its specific serum are substituted for the hog cholera bacillus, essentially the same results are obtained, as will be brought out in Experiment 5.

*Experiment 5.*—Normal rabbit serum and rabbit serum containing agglutinin for *B. abortus* were diluted in 4 parts of NaCl solution. The diluted serum was distributed in amounts of 3 cc. in a series of tubes, and the tubes were then heated at 70°, 75°, 80°, 85°, and 90°C. for 20 minute intervals. A heavy suspension of *B. abortus* was added to each tube and all were incubated 1½ hours. A tube of bacterial suspension in salt solution was also incubated. The contents of all tubes were agitated at frequent intervals. After incubation, an excess of salt solution was added and the tubes centrifuged. The bacteria were then resuspended in salt solution and tested with unheated specific serum. The results of this treatment on subsequent agglutination are given in Table IV. The controls, where normal rabbit serum was used, failed to show that such treatment influenced subsequent agglutination and are for this reason omitted from the table.

The *Bacillus abortus* serum on the whole behaves when heated much like the other agglutinin. There is however this difference, that the

substance in the abortion bacillus antiserum is more resistant to heat since sufficient combination existed even after heating the antiserum to 80°C. to prevent agglutination on addition of unheated immune serum. Immune sera heated to 85°C. and 90°C. have no inhibiting effect.

That there is an actual fixation between bacterial cell and serum protein can again be shown by noting the effect when rabbit serum precipitin is added to the bacterial suspension which had first been treated with heated agglutinin. This has been brought out in Experiment 6.

TABLE IV.

*The Behavior of Bacillus abortus after Treatment with Heated Immune Serum.*

| | Dilutions of unheated immune rabbit serum | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | Control |
| Bacilli exposed to diluted serum heated at °C. | | | | | | | | |
| 70 | − | − | − | − | − | − | − | − |
| 75 | − | − | − | − | − | − | − | − |
| 80 | − | − | − | − | − | − | − | − |
| 85 | C | C | ++++ | +++ | ++ | + | + | − |
| 90 | C | C | ++++ | +++ | ++ | + | + | − |
| Control—bacilli exposed to NaCl | C | C | C | C | ++ | + | + | − |

*Experiment 6.*—This experiment is virtually a repetition of Experiment 3 except that *B. abortus* and its agglutinin were used. The procedure was the same. The results are given in Table V.

As in Experiment 3, Table V indicates that antiserum heated at 75°C. actually combines with the bacterial cell in such quantities that agglutination results when a precipitin specific for rabbit serum is added. The blood serum proteins are not dislodged by washing. The combination is less marked when the immune serum is first heated at 80°C., nevertheless the reaction is more intense than that occurring after suspension in immune serum heated at 85°C. and 90°C., or normal rabbit serum heated at the same temperatures.

It was possible by complement fixation tests to further confirm the findings that immune serum heated to 75°C. for 20 minutes completely

deviates the complement in the presence of antigen. That heated to 80°C. markedly deviates the complement, while there is only a slight deviation with serum heated at 85°C., and none with that heated at 90°C.

## DISCUSSION.

It is true that bacteria when first treated with immune serum heated at various temperatures may or may not agglutinate on subsequent

TABLE V.

*The Effect of Rabbit Serum Precipitin on Bacillus abortus First Treated with Heated Immune Serum.*

| | Cc. of rabbit serum precipitin | | | | | |
|---|---|---|---|---|---|---|
| | 1:50 | 1:100 | 1:200 | 1:500 | 1:1,000 | Control |
| Bacilli treated 1½ hr. with immune serum diluted 1:4 first heated for 20 min. at °C. | | | | | | |
| 75 | +++ | ++++ | ++ | + | + | − |
| 80 | +++ | ++ | − | − | − | − |
| 85 | + | + | − | − | − | − |
| 90 | + | ± | − | − | − | − |
| Bacilli treated with diluted normal rabbit serum first heated at 75°, 80°, 85°, and 90°C. | + | ± | − | − | − | − |
| Bacilli treated with NaCl solution | − | − | − | − | − | − |

exposure to agglutinin. When the hog cholera bacillus antiserum was heated to 75°C. for 20 minutes, the bacilli exposed to its action failed to agglutinate when subsequently treated with unheated immune serum. When the first test serum is heated at 80°C., relatively little inhibiting effect is encountered. The reaction is a specific one, since it is not encountered when normal serum is used in a similar manner. In this regard *Bacillus abortus* acts much like the hog cholera bacillus when similar experiments are performed. However, *Bacillus abortus* serum heated at 80°C. is still capable of preventing further agglutination of the bacilli.

Ehrlich's conception is that two substances produce agglutination,—one is a combining body which is thermostabile, and the other produces clumping and is thermolabile. The latter is converted into agglutinoid

by heat. The combining substance is uninjured by heat and enters into combination with the bacterial cell, thus preventing union on the addition of fresh agglutinin. The evidence that I have submitted while not conclusive is at least suggestive that the reaction may be explained on other grounds.

In the first place, the temperature at which the earlier investigators heated their sera (65°C. and 70°C.) has in my hands always agglutinated the organisms. Furthermore, they worked with undiluted serum and it is possible that agglutination failed to take place because of an excess of colloid, *i.e.*, they may have been working in the prozone. When the evidence in regard to the hog cholera bacillus and its agglutinin is analyzed more carefully it is evident that heating at 75°C. for 20 minutes is insufficient to destroy the agglutinin. In fact, the bacilli will agglutinate with such serum provided the incubation is long enough and if interfering mechanical factors are avoided. It has been shown that this is the case by several investigators. The writer (4) brought out the fact that as the temperature increases the agglutinin content declines, at first gradually, until 75°C. is reached. The break is sharp at 80°C., although a little antibody can be detected even after heating to 90°C. There remains, then, in the case of this serum considerable agglutinin after heating the serum to 75°C. It is suggested that this agglutinin combines with the bacterial cell, but as the result of mixing, too short incubation, or for other reasons, the combination is not quantitatively sufficient to produce the phenomenon of agglutination. It may be that agglutinin is so modified during heating at certain temperatures that, although still capable of combining with the organisms, the usual phenomenon of such combination (agglutination) fails to take place. The union is apparently sufficient to prevent further union when fresh immune serum is added. That union actually takes place between the bacterial cell and the serum proteins of certain heated sera is definitely shown by the reaction when a specific precipitin is added, since it is known that agglutination results when bacteria or collodion particles are sensitized to proteins on addition of specific precipitin. As further evidence the behavior of the heated serum in the complement fixation tests is suggestive. In both cases cited the sera heated sufficiently to prevent the secondary agglutination after the addition of fresh serum always deviated the complement. When the sera were heated to higher

temperatures and failed to prevent the secondary agglutination, then relatively little or none of the complement was deviated.

The evidence for *Bacillus abortus* agglutinin is not so clear, although it is known that the agglutinin will resist heating to 70°C. for 20 minutes. Serum heated above this point no longer causes agglutination. Nevertheless when the immune serum is heated to 75°C. and 80°C. it is still capable of preventing a second agglutination. It is clear, however, that actual union of bacterial cell and serum proteins occurs even after heating the immune serum to these temperatures. It is also true that such sera were able to bind complement.

In the light of the experimental evidence the burden of proof that agglutinoid actually exists remains to be established, since the blocking of secondary agglutination can be explained on the basis of residual antibody.

## SUMMARY.

It is shown that when dilute rabbit serum rich in agglutinin for the hog cholera bacillus is heated at 75°C. for 20 minutes and the bacilli incubated with the heated serum, agglutination fails to result on the addition of unheated immune serum. When the immune serum is first heated to 80°C., it no longer greatly inhibits secondary agglutination when the organisms are exposed to fresh agglutinin. The abortion bacillus agglutinin acts in a similar manner except that the immune serum must be heated above 80°C. for 20 minutes to prevent the second agglutination. The reactions are specific since control experiments with normal rabbit serum heated at various temperatures failed to influence further agglutination. It has also been shown by precipitation tests that there is definite fixation of serum proteins and bacterial cells with the heated sera which would prevent subsequent agglutination. Furthermore, heated antiserum which would prevent the secondary agglutination still possessed the property of deviating complement in a hemolytic series.

## BIBLIOGRAPHY.

1. Eisenberg, P., and Volk, R., *Z. Hyg. u. Infectionskrankh.*, 1902, xv, 155.
2. Beyer, H. G., and Reagh, A. L., *J. Med. Research*, 1904, xii, 313.
3. Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 627.
4. Jones, F. S., *J. Exp. Med.*, 1927, xlvi, 291.
5. Jones, F. S., *J. Exp. Med.*, 1927, xlvi, 303.

# A COMPARISON OF THE SPIROCHETE OF YELLOW FEVER (LEPTOSPIRA ICTEROIDES NOGUCHI) WITH THE LEPTOSPIRA OF WEIL'S DISEASE.

By ERICH MARTINI, M.D.

*(From the Hygienic Laboratory of Samper and Martinez, Bogota, Colombia, South America.)*

(Received for publication, July 7, 1927.)

In the year 1923, yellow fever prevailed in Bucaramanga, the capital of the Department of South Santander in Colombia, South America. The diagnosis, which was made at the time by a group of local physicians,[1] was confirmed by a commission of the International Health Board of the Rockefeller Foundation.[2] This commission conducted its work in our laboratory at Chapinero (Bogota). The confirmation was made serologically by Pfeiffer tests in guinea pigs furnished by us, cultures of *Leptospira icteroides* having been forwarded by Dr. Noguchi. It then became an aim of the laboratory to be prepared to determine a diagnosis of yellow fever by Pfeiffer tests. For the purpose we made an attempt to grow our own cultures of *Leptospira icteroides* from the only guinea pig that survived the Pfeiffer tests of the commission.

The animal (Guinea Pig 1) had been inoculated intraperitoneally by Dr. Pothier on May 16, 1923, with a rich culture of *Leptospira icteroides* (grown on Noguchi medium) together with human serum from a suspected case of yellow fever. On May 24, 1923, 8 days later, heart's blood was taken from the inoculated animal and placed in Ungermann medium, containing rabbit serum inactivated for 30 minutes at a temperature of 58–60°C. and covered with a protective layer of paraffin. On June 9, 1923, the culture showed an abundant growth of lepto-

[1] Drs. Daniel Peralta, Martin Carvajal, Francisco Pradilla Gonzalez, Andres Gomez, Julio Valdivieso, Roberto Serpa, and Louis Ardila Gomez, who in a publication issued in April, 1923, substantiated their findings by clinical histories.

[2] Drs. J. H. White, Pothier, and Wenceslao Pareja, who were sent in response to a request made by the Government of Colombia, through the Director of Hygiene, Dr. Pablo Garcia Medina.

spira, and we were, therefore, in a position to undertake serological tests for yellow fever.

In our subsequent work we employed the pure or a modified form of the Ungermann culture medium and adopted the following procedure. During the early months, we made weekly transfers to guinea pigs. Certain animals were inoculated with *Leptospira icteroides* (Noguchi), and others, for purposes of comparison, with *Leptospira icterohæmorrhagiæ* (Weil's disease). Series of cultures were made in both instances, at first with heart's blood, or suspensions of liver and kidney. Later on, cultures were inoculated only with heart's blood. In every case, the pathogenic material was taken on the day of maximal fever and cultured promptly.

With the cultures thus obtained, guinea pigs were again inoculated intraperitoneally, and from the heart's blood of the infected animals, other cultures were made for the inoculation of fresh guinea pigs, and so on. It was observed that the virulence of the spirochetes decreased gradually in the process of passage, so that finally, in August, 1923, after about 3 months of transfer, no animals died of the infection, although large numbers of spirochetes were injected. During the subsequent months, an animal would now and then exhibit a slight degree of the characteristic icterus, but notwithstanding the high fever (up to 41°C.), all survived the infection. Ultimately no jaundice was perceptible, but cultures could still be obtained from the heart's blood taken at the height of the fever. As Lebredo of Havana in his publications reported that he had observed viability up to 7 months of *Leptospira icteroides* in cultures made on the Noguchi medium, we continued to make inoculations of guinea pigs, and subsequent cultures with the heart's blood, at intervals of from 3 to 6 months, and are today—more than 3 years later—still in possession of living cultures of this particular strain.

*Leptospira icterohæmorrhagiæ*, which by our present methods cannot be distinguished morphologically from *Leptospira icteroides*, was likewise inoculated and cultured. No means of differentiation between the two types of leptospira was apparent at first. But later on, differences in respect to the duration of life of the two types of cultures became evident, and these differences increased as modifications in the culture media were undertaken.

The cultures of *Leptospira icterohæmorrhagiæ* during the early months were grown on Ungermann medium; later on, we employed for one half of the cultures a medium made of inactivated rabbit serum and physiological salt solution in the proportion of 1: 3; and finally the heart's blood only was used in physiological salt solution, a process which appears to have been first employed by Uhlenhuth and Zuelzer. In a number of instances, cultures were made with rabbit serum which had not been inactivated, but this method was abandoned, since as a rule

the results thus obtained were not as good as when the original Ungermann method was employed.

About 100 cultures of each of the two types—*Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*—were made according to the original Ungermann technique; about an equal number of cultures with inactivated rabbit serum and physiological salt solution, 1:3; and 50 cultures each in physiological salt solution. In a few cases, a 1:3 mixture of uninactivated rabbit serum and physiological salt solution was used. The employment of the last mentioned medium did not give as consistent results as the mixture made with inactivated rabbit serum, although one of these *Leptospira icteroides* cultures proved viable after 2 years and 5 months.

It is noteworthy that when a small quantity of rabbit blood was added to the mixture of rabbit serum and physiological salt solution, the growth of *Leptospira icterohæmorrhagiæ* was abundant, while on the other hand the cultures of *Leptospira icteroides* in this medium did not as a rule show a great increase of growth. But neither of the two types of spirochete was viable for particularly long periods of time when grown in this medium.

Tables I and II show the growth periods of the two types in various media, the longest periods being recorded. The terms *Leptospira icteroides* and *icterohæmorrhagiæ* have been employed because they are current in Central and South America.

The conclusions to be drawn from these tables are:

1. In a medium made of physiological salt solution, in which *Leptospira icteroides* is known to grow less vigorously than *Leptospira icterohæmorrhagiæ*, the longest period of growth for *Leptospira icteroides* was $6\frac{1}{2}$ months, *i.e.* a period about one-half to two-thirds that of *Leptospira icterohæmorrhagiæ*, which was alive after 11 months.

2. In the Ungermann culture medium, *Leptospira icteroides* lived nearly a year longer (3 years in all) than *Leptospira icterohæmorrhagiæ* (2 years and 1 month).

3. With the use of inactivated rabbit serum and physiological salt solution, 1:3, *Leptospira icteroides* survived six times as long (3 years and $6\frac{2}{3}$ months) as *Leptospira icterohæmorrhagiæ* ($6\frac{2}{3}$ months), although the growth of the latter was abundant.

In judging these cultivation experiments, account must be taken of the fact that a comparison has been made in each case with but a single strain of the two types of leptospira. In order to arrive at

TABLE I.

*Leptospira icteroides.*

| No. | Guinea pig | Dates cultures were made | Medium | Date last examined | No. of surviving cultures | Viability | |
|---|---|---|---|---|---|---|---|
| | | | | | | *yrs.* | *mos.* |
| 1 | 2 | 9/24/24 | Isotonic salt solution | 11/ 6/24 | 1 | | $1\frac{1}{2}$ |
| 2 | 3 | 9/24/24 | " " " | 11/ 6/24 | 1 | | $1\frac{1}{2}$ |
| 3 | 4 | 5/26/24 | " " " | 9/ 8/24 | 1 | | $3\frac{1}{2}$ |
| 4 | 5 | 12/20/24 | " " " | 5/31/25 | 1 | | $6\frac{1}{2}$ |
| 5 | 6 | 5/14/25 | Blood; inactivated rabbit serum, isotonic salt solution | 3/ 5/26 | 1 | | $9\frac{1}{2}$ |
| 6 | 7 | 1/25/24 | Inactivated rabbit serum, isotonic salt solution | 12/23/24 | 1 | | 11 |
| 7 | 8 | 3/12/26 | Ungermann | 5/27/27 | 1 | 1 | 2 |
| 8 | 2 | 9/24/24 | Inactivated rabbit serum, isotonic salt solution | 3/11/26 | 1 | 1 | 6 |
| 9 | 9 | 9/11/23 | Ungermann | 5/19/25 | 1 | 1 | 8 |
| 10 | 10 | 3/10/24 | Inactivated rabbit serum, isotonic salt solution | 3/ 5/26 | 1 | 1 | 11 |
| 11 | 9 | 9/11/23 | Ungermann | 10/ 5/25 | 2 | 2 | |
| 12 | 11 | 12/ 1/23 | " | 3/ 5/26 | 1 | 2 | 3 |
| 13 | 12 | 12/22/24 | *Active* rabbit serum, isotonic salt solution | 5/27/27 | 1 | 2 | 5 |
| 14 | 13 | 12/ 1/23 | Ungermann | 12/10/26 | 1 | 3 | |
| 15 | 7 | 1/25/24 | Inactivated rabbit serum, isotonic salt solution | 5/27/27 | 1 | 3 | 4 |
| 16 | 14 | 11/ 5/23 | Inactivated rabbit serum, isotonic salt solution | 5/27/27 | 1 | 3 | $6\frac{2}{3}$ |

TABLE II.

*Leptospira icterohæmorrhagiæ.*

| No. | Guinea pig | Dates cultures were made | Medium | Date last examined | No. of surviving cultures | Viability | |
|---|---|---|---|---|---|---|---|
| | | | | | | *yrs.* | *mos.* |
| 1 | 15 | 12/20/24 | Isotonic salt solution | 5/19/25 | 2 | | 6 |
| 2 | 16 | 5/26/24 | Inactivated rabbit serum, isotonic salt solution | 12/17/24 | 1 | | $6\frac{2}{3}$ |
| 3 | 17 | 8/22/24 | Isotonic salt solution | 5/19/25 | 1 | | 9 |
| 4 | 18 | 5/26/24 | " " " | 4/29/25 | 2 | | 11 |
| 5 | 19 | 11/30/23 | Ungermann | 5/19/25 | 1 | 1 | $5\frac{1}{2}$ |
| 6 | 20 | 11/30/23 | " | 5/19/25 | 1 | 1 | $5\frac{1}{2}$ |
| 7 | 21 | 3/10/24 | " | 3/ 6/26 | 1 | 2 | |
| 8 | 22 | 1/22/24 | " | 2/26/26 | 1 | 2 | 1 |

really conclusive results, it will be necessary to make comparative studies of many strains of both types. The above mentioned results, however, may serve to indicate a direction for further investigation, since they suggest a possible means of differentiation of the organisms.

As the course of the infection with *Leptospira icteroides* in the experimental guinea pig has been minutely described by Noguchi, we shall not take it up here. The clinical history and the pathological anatomy of the animals have moreover been extensively dealt with by W. H. Hoffmann of Havana. On the basis of careful experimental work, the last mentioned author maintained for a long time a skeptical attitude regarding the etiological significance of *Leptospira icteroides* in yellow fever, because of the clinical and pathological similarities to *icterohæmorrhagiæ* infections in guinea pigs. Finally, however, even Hoffmann, on the basis of his studies of human yellow fever cases, of the Pfeiffer reaction, and of the specificity of the yellow fever vaccine and serum, appears no longer to doubt that *Leptospira icteroides* of Noguchi is the active, causative agent of yellow fever.

With regard to *Leptospira icteroides* from the guinea pig, the following points which Noguchi has already elucidated appear to be significant:

As a rule, the temperature attains its greatest height (40–41°C. and over) from the 4th to the 7th days after inoculation, while the peak is only occasionally attained on the 3rd day, or on the 8th to 11th days. Sometimes, however, the temperature may go as high as 40°C. on even the 14th day after inoculation. Temperatures up to 41°C. and over, are, however, not necessarily indicative of a severe infection. Guinea Pigs 23, 24, and 11 inoculated with *Leptospira icteroides* recovered completely after very high temperature rises. The first two animals showed the typical icterus. On the other hand, guinea pigs may succumb in a few days with a temperature of less than 40°C., the temperature suddenly falling below normal. In such cases, icterus, typical cutaneous hemorrhages, epistaxis, necrosis of the liver, and lime casts in the kidneys were all noted.

It is desirable to take the material for cultivation—heart's blood, suspension of liver or kidney—at the height of the fever, if possible when the temperature has reached 40° or over, and to place it immediately in the culture medium. According to our experience, this high body temperature, which is most favorable for insuring a vigorous culture growth, continues for only 1 or 2 hours and does not progress after the initial rise has been attained. Particularly is this the case in infections produced with avirulent cultures. Hence it is necessary in such instances to watch the animals closely in order to utilize a favorable opportunity for obtaining blood or other material.

The earliest period at which we were able to obtain positive results from cultures was 5 days after planting. As a rule, however, an abundant growth could not be obtained before about the 10th day. It happened not infrequently that culture media which appeared to be sterile for a number of weeks began to show signs of growth after about a month.

The temperature most favorable to the growth of cultures ranged between 24° and 34°C., but cultures kept at temperatures below 30°C. retained their viability for a longer time, for several weeks at least. If kept between 22° and 24°C., the organisms remain viable for a long period.

## CONCLUSIONS.

The most noteworthy point observed in our studies is the extraordinary duration of life and the relatively meagre requirements for sustenance of *Leptospira icteroides*. It is conceivable that under natural conditions opportunities might arise for the prolonged existence of *Leptospira icteroides*, so that possibly after a lapse of years, the disease might reappear without introduction from outside. At any rate, the great viability of *Leptospira icteroides* must be considered in this connection.

A decrease in the virulence of the leptospira does not rule out the possibility of a renewed outburst of yellow fever, since Uhlenhuth and Zuelzer have shown that it is possible to set up Weil's disease in guinea pigs by means of apparently saprophytic spirochetes obtained from drinking water, when the virulence of these organisms has been artificially heightened.

In conclusion the writer takes pleasure in expressing his appreciation for the collaboration in this work of Dr. Bernardo Samper Sordo.

# THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

## V. Phenomena of Passive Immunity in Blocked and Splenectomized Mice.

By C. W. JUNGEBLUT, M.D.*

*(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.)*

(Received for publication, November 16, 1927.)

### INTRODUCTION.

The study of the reticulo-endothelial system in its relation to the various phenomena of immunity and anaphylaxis has during the last few years engaged an increasing number of investigators. Several extensive reviews (1–6) on this subject have recently appeared to which the reader is referred for detailed information. In this country the work of Gay and his associates has done much to bring to light the important rôle of the reticulo-endothelial cells in the formation of antibodies, particularly with reference to the mechanism of local immunity. Valuable information on the significance of the reticulo-endothelial system in the various immunity reactions has been derived from an application of the so called blockade method, either alone or together with splenectomy, to the particular problem under consideration. Without discussing the merits of this procedure as a means of eliminating or temporarily invalidating the normal physiological action of the respective cells, let it suffice to state that methods of blockade have been of distinct value in the experience of many workers, although it must be conceded that others have not been able to secure such uniform results.

The problem as to the site of antibody production may be approached (1) by measuring *in vitro* the antibody titer of blocked or splenectomized animals immunized with non-living antigens, and (2) by following the course of various acute and chronic infections in animals so treated. Observations of the latter kind have up to the present been less numerous. Besides the attempts which have been made to break the natural immunity of certain species to given infections and intoxications by endothelial blocking or splenectomy (7), the work of Tsuda (8), Hoen, Tschertkow and Zipp (9), Ledingham (10), Wright (11), Singer and Adler (12), Oerskov (13) and Bass (14) should be mentioned in this

* Fellow in Medicine, National Research Council.

connection. In the case of relapsing fever in mice the apparent lack of antibody production after blockade and splenectomy was recently demonstrated by the writer (15). These findings were fully corroborated by the contemporaneous work of Kritschewsky and Rubinstein (16), and further amplified by the more recent studies of Lisgunova and Butjagina (17). On the other hand, Feldt and Schott (18) and Bruynoghe and Collon (19) did not observe any difference in the course of this infection between blocked or splenectomized and normal mice. The reason for this discrepancy is not altogether clear. That one may not always be justified in generalizing from observations on one type of infection is illustrated by the fact that in some unpublished experiments, the writer noted no material difference in the character or course of syphilitic and tuberculous infection in blocked and splenectomized mice and guinea pigs. This refers to the manifestations and distribution of the clinical lesions in general. Likewise, in rabbits infected with *Trypanosoma equiperdum*, the number of parasites in the blood remained unaltered after the administration of massive intravenous doses of Indian ink. Mention should also be made that the parasites disappeared critically from the circulation on or about the 6th day of the disease—a phenomenon which was described by Mutermilch in 1911 (20)—with the same regularity in blocked animals as they did in controls.

The introduction of blockade methods in the endeavor to analyze phenomena of passive immunity has only recently been made by Neufeld and Meyer (21) and Meyer (22), although the earlier studies of Lippmann (23) had already served to emphasize the significance of the mobile phagocytic cells for the establishment of passive immunity in pneumococcus and streptococcus infection. The first mentioned authors were unable to note any difference between blocked or splenectomized and normal mice with reference to the protective action of antipneumococcus serum against Pneumococcus Type I infection in this species. Meyer (22), therefore, concludes that the destruction of the pneumococci by the immune serum is essentially a humoral process, not primarily involving the participation of phagocytosis.

In view of the many facts which suggest the important function of the phagocytic cells, both mobile and sessile, in the elimination of pneumococci from the circulation of the immune animal, it appeared desirable to employ the experimental methods of blockade or splenectomy in a study of the passive immunity against this infection. At the same time it was considered of particular interest to carry on an investigation, based on similar principles, into the nature of passive immunity to tetanus toxin. The disease processes mentioned were chosen on the ground that they represent examples of two types of immunity, that is, antibacterial and antitoxic, which are believed to differ fundamentally in their mechanism.

## EXPERIMENTAL WORK.

### *A. Experiments on the Effect of Blockade and Splenectomy on the Passive Immunity in Pneumococcus Type I Infection.*

These experiments were carried out in normal white mice weighing from 18 to 24 gm. The pneumococcus strain was a highly virulent Type 1 culture (N5); the immune serum, an Antipneumococcus Type 1 horse serum produced for therapeutic purposes. Both the bacterial strain and the serum were obtained from the Division of Laboratories and Research of the New York State Department of Health, Albany, New York, through the courtesy of Dr. Wadsworth and Miss Kirkbride.

Before studying the effect of blockade and splenectomy on the protective action of antipneumococcus serum against pneumococcus infection in mice, it has seemed advisable, for purposes of control, to obtain data on the influence of the above mentioned experimental procedures on the course of untreated pneumococcus infection in this species. It was found that neither a preceding intravenous injection of India ink (1 cc. of 1:15 dilution) nor the removal of the spleen, nor a combination of both these procedures was capable of altering the course of Pneumococcus Type I infection in mice. This statement is made with reference to the size of the minimum fatal dose, the length of time the animals lived and the time of appearance of the pneumococci in the blood stream after intraperitoneal inoculation. Essentially the same conditions prevailed when the intravenous route was chosen as the mode of infection, with the exception, however, that death was very much delayed in both the experimental animals and controls. Doses of 0.000001 cc. and 0.001 cc., if given intravenously, caused death only after 5 and 3 days respectively, while the same doses upon intraperitoneal inoculation killed the animals promptly in from 24 to 48 hours.

The influence of blockade and splenectomy on the action of Antipneumococcus Type 1 serum in Pneumococcus Type I infected mice was studied next. The experiments were carried out by injecting intraperitoneally mixtures of a constant amount of serum (0.1 cc.) and varying doses of an 18 hour culture (0.1, 0.2, 0.3 cc.), in a uniform volume of 0.5 cc. each, into three groups of mice. The first group represented normal controls, the second had received 1 to 2 hours before an intravenous injection of India ink (1 cc. of 1:15 dilution) while the last group had been splenectomized 2 days previously. For each mixture, duplicate animals were run, and the final results of each test were based on an arbitrary 4 day observation. Virulence controls (0.000001 cc. and 0.00000001 cc.) accompanied each test. Instead of recording each individual experiment, the results of five tests, carried out on separate dates, are chartered together. Although there are disadvantages to this method of evaluating the results, it is possible to obtain average figures without permitting the irregularities in virulence and the variability in individual susceptibility to obscure the fundamental results in the experiment.

As may be seen from Table I, the protective action of Antipneumococcus Type I serum against the homologous infection was definitely lower in mice injected with India ink as compared with the normal controls. While 0.1 cc. of serum protected fully 100 per cent of normal mice against 0.1 cc. of culture, only 40 per cent of the blocked mice lived on the 4th day after the test. On the other hand, when splenectomy preceded the test shortly before, there seemed to be no

TABLE I.

*Action of Antipneumococcus Type I Serum against Pneumococcus Type I Infection in Normal, Blocked and Splenectomized Mice.*
*(Combined Results of Five Tests.)*

| No. of animals | Preparation | Mixtures (injected intraperitoneally) | | Result | | Protection |
|---|---|---|---|---|---|---|
| | | Serum | Culture | Survived | Died | |
| | | *cc.* | *cc.* | | | *per cent* |
| 10 | Normal | 0.1 | 0.1 | 10 | 0 | 100 |
| " | Blocked | " | " | 4 | 6 | 40 |
| " | Splenectomized | " | " | 9 | 1 | 90 |
| " | Normal | " | 0.2 | 6 | 4 | 60 |
| " | Blocked | " | " | 3 | 7 | 30 |
| " | Splenectomized | " | " | 5 | 5 | 50 |
| " | Normal | " | 0.3 | 4 | 6 | 40 |
| " | Blocked | " | " | 1 | 9 | 10 |
| " | Splenectomized | " | " | 4 | 6 | 40 |
| 5 | Normal | — | 0.000001 | 0 | 5 | 0 |
| " | " | — | 0.00000001 | " | " | " |

material diminution in the degree of passive immunity. Similar conditions were found to prevail in the case of other quantitative combinations of serum and culture. In a few instances, the serum was given prophylactically at various intervals (24, 48 and 72 hours) before the infection. Although the results available are too few upon which to base definite conclusions, they indicate that the protective action of a prophylactic dose of serum against subsequent infection was diminished still more in blocked animals.

### *B. Experiments on the Effect of Blockade and Splenectomy on the Passive Immunity to Tetanus Toxin.*

These experiments were carried out in normal white mice, weighing from 18 to 24 gm. The tetanus toxin was a recently prepared broth toxin which at the time of its preparation had a minimum fatal dose for guinea pigs of 0.0001 cc. The antitoxin was a refined and concentrated pseudoglobulin prepared from antitoxic horse plasma, and contained 800 units per cc. Both the toxin and antitoxin were obtained from Dr. Wadsworth and Miss Kirkbride of the New York State Department of Health, to whom my best thanks are due.

In preliminary experiments, the potency of this toxin for mice was titrated in normal animals and in mice which had (1) received one intravenous blocking injection of India ink 1 to 2 hours before the test; (2) which had been splenectomized 2 days previously; (3) which had been subjected to splenectomy and blockade. There was no difference in the lethal action of the toxin for normal and experimental animals, the minimum fatal dose being with each group 0.001 cc. after subcutaneous injection, and 0.0005 cc. after intravenous injection.

The influence of blockade and splenectomy on the neutralization of tetanus toxin by specific antitoxin and on the protection afforded by a preceding dose of antitoxin against subsequent intoxication was studied. The first part of these experiments was carried out by injecting subcutaneously, as well as intravenously, mixtures of a constant amount of toxin (two hundred minimum fatal doses = 0.2 cc.) and varying amounts of antitoxin (0.002, 0.001, 0.0005, 0.00025 cc.) into normal and experimental mice. The mixtures contained the toxin and antitoxin in a uniform volume of 0.5 cc. each and had been held 1 hour at room temperature before the injection was made. Duplicate animals were run for each toxin-antitoxin mixture; the results of two tests are recorded together in Table II.

Table II shows clearly that the various toxin-antitoxin mixtures were toxic or non-toxic for experimental animals to the same extent as they were for the normal controls, the minimum dose of antitoxin which neutralized exactly two hundred minimum fatal doses in this case being in either group 0.001 cc. These results then demonstrate that, after union between toxin and antitoxin has taken place *in vitro*, the reaction on the part of the animal represents merely a registration of the toxicity of a given mixture and the physiological effect is in direct proportion to the amount of free toxin present. This process, in accordance with the previous findings, does not seem to involve primarily the reticulo-endothelial cells in its accomplishment.

Further experiments were planned to provide for a union of toxin

and antitoxin *in vivo*, allowing sufficient time for a general distribution of the antitoxin in the organism. Experimental animals, blocked by an intravenous injection of India ink as described before, together with

TABLE II.

*Titration of Tetanus Toxin and Antitoxin in Normal, Blocked and Splenectomized Mice.*

(*Combined Results of Two Tests.*)

| No. of animals* | Preparation | Mixtures (injected either subcutaneously or intravenously) | | Results |
|---|---|---|---|---|
| | | Toxin | Antitoxin | |
| | | cc. | cc. | |
| 4 | Normal | 0.2 | 0.002 | All four alive 4th day |
| " | Blocked | " | " | " " " " " |
| " | Splenectomized | " | " | " " " " " |
| " | Blocked and splenectomized | " | " | " " " " " |
| " | Normal | " | 0.001 | " " " " " |
| " | Blocked | " | " | " " " " " |
| " | Splenectomized | " | " | " " " " " |
| " | Blocked and splenectomized | " | " | " " " " " |
| " | Normal | " | 0.0005 | 2 dead 2nd, 2 dead 3rd day |
| " | Blocked | " | " | " " " " " " " |
| " | Splenectomized | " | " | 3 " " 1 " " " |
| " | Blocked and splenectomized | " | " | 2 " " 2 " " " |
| " | Normal | " | 0.00025 | All four dead 2nd day |
| " | Blocked | " | " | " " " " " |
| " | Splenectomized | " | " | " " " " " |
| " | Blocked and splenectomized | " | " | " " " " " |

* In each group of four animals, two received the respective toxin-antitoxin mixture subcutaneously, two intravenously.

an equal number of normal controls were given a constant amount of antitoxin (0.01 cc.) intraperitoneally, and after varying intervals, *i.e.*, 24, 48 and 72 hours, received by the same route graded doses of toxin (0.2, 0.1, 0.05, 0.01 cc.) in order to determine the degree of passive

immunity. The results, which are shown in Table III, demonstrate a marked difference between the experimental animals and controls.

TABLE III.

*Protective Effect of a Preceding Dose of Tetanus Antitoxin against Subsequent Intoxication in Normal and Blocked Mice.*

| No. of animals | Preparation | Antitoxin injected intraperitoneally | Toxin injected intraperitoneally | Interval* | Result | |
|---|---|---|---|---|---|---|
| | | | | | Survived | Died |
| | | *cc.* | *cc.* | *hrs.* | | |
| 1 | Normal | 0.01 | 0.2 | 24 | 1 | 0 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | " | " |
| " | " | " | 0.01 | | " | " |
| " | Blocked | " | 0.2 | 24 | 0 | 1 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | 1 | 0 |
| " | " | " | 0.01 | | " | " |
| " | Normal | " | 0.2 | 48 | 1 | 0 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | " | " |
| " | " | " | 0.01 | | " | " |
| " | Blocked | " | 0.2 | 48 | 0 | 1 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | 1 | 0 |
| " | " | " | 0.01 | | " | " |
| " | Normal | " | 0.2 | 72 | 1 | 0 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | " | " |
| " | " | " | 0.01 | | " | " |
| " | Blocked | " | 0.2 | 72 | 0 | 1 |
| " | " | " | 0.1 | | " | " |
| " | " | " | 0.05 | | " | " |
| " | " | " | 0.01 | | " | " |

* This interval represents the elapsed time between the injection of the toxin and the antitoxin.

While, under the conditions of the test, a prophylactic dose of 0.01 cc. of antitoxin was capable of protecting normal mice against 0.2 cc. of

toxin for fully 72 hours, the blocked mice after an interval of 24 and 48 hours were protected only against 0.05 cc. of toxin and after a period of 72 hours no protection at all was noted against even 0.01 cc., the smallest dose of toxin employed.

## DISCUSSION.

In discussing the observations made in this study, the fundamental difference between the reaction of the antibacterial serum, on the one hand, and the reaction of the antitoxin, on the other, with their specific antigens, becomes at once apparent. Both, pneumococcus infection and tetanus intoxication in mice, represent examples in which agents of maximum virulence or toxicity are introduced into a host of maximum susceptibility. Interference with the cellular defensive mechanism of the host, such as is brought about by blockade of the reticulo-endothelial system or splenectomy, under such conditions, is not apt to further decrease this minimum of resistance. In accordance with this consideration, it was found that both disease processes were not altered appreciably in their course by the above mentioned experimental procedures. The results obtained from this part of the work at the same time contradict an objection, which has been raised by some authors against the value of blockade as a means for the selective elimination of the antibody-producing reticulo-endothelial cells. It has been claimed that the injection of foreign colloidal material is a procedure which in a general, non-elective fashion lowers the vitality of the animal and consequently reduces its capacity to respond to an antigenic stimulus. The present experiments show that in processes, the acute course of which does not allow for antibody production, neither blockade nor splenectomy affect the general vitality of the experimental animal, as is the case where other experimental methods are used, such as injection of certain chemical poisons, exposure to actinic rays, removal of the glands of internal secretion, starvation, diet deficiencies, etc.

The action of antibacterial serums in general on their respective infections has long been recognized to involve the active participation of the passively immunized individual in the final destruction of the respective microorganisms, quite apart from any possible direct interaction between antibodies and antigen. Such immune reactions differ

from the toxin-antitoxin neutralization not only in the fact that no deleterious reaction *in vitro* is demonstrable without the participation of living tissue cells or of ferments, but also by their failure to follow the laws of multiple proportions. The mere fact that the protection afforded by an antibacterial serum against the corresponding infection is definitely limited, the protective action of increasing amounts of serum not rising proportionately against increasing doses of culture, suggests the existence in the animal of a third factor, which determines to a large extent the outcome of the test. Furthermore, the importance of such individual disposition is reflected by the apparent irregularities in the results of protection tests with streptococcus and pneumococcus sera, while the respective infections alone are marked by their uniform and regular course. In the case of Pneumococcus Type I infection, it has long been realized that the protective effect of antipneumococcus serum against this infection is not due to any bactericidal action of the serum, but must be linked closely with active participation of the phagocytic cells of the treated animal (bacteriotropins). It is, therefore, to be expected that any interference with the physiological integrity of the reticulo-endothelial system must of necessity lower the protective action of the immune serum. The results obtained in this study, which clearly demonstrate a lowered protective effect of Antipneumococcus Type I serum against Pneumococcus Type I infection in blocked mice, in the writer's opinion, may be conveniently interpreted in conformity with the outlined theoretical considerations. In this connection it is of interest to draw briefly attention to the work of Kritschewsky and Meersohn (24), Kolpikow (25) and Feldt and Schott (18), as well as to our own observations (15), which have shown that the chemotherapeutic effect of salvarsan on relapsing fever and trypanosome infection in mice likewise depends on the presence of an intact reticulo-endothelial system. The writer has suggested that the marked discrepancy in the case of salvarsan between the lack of action *in vitro* and the high parasiticidal power of the drug in the infected animal on the corresponding organisms may possibly be explained by a hitherto unknown function of the reticulo-endothelial cells, which may account for the transformation of the inactive preparation into the active derivative. Earlier observations of Rosenthal and Spitzer (26) on the relation of

the trypanocidal action of human serum to the reticulo-endothelial system would seem to strengthen such an hypothesis.

Contrasting the mechanism of passive immunity, induced by the so called antibacterial sera, with an analysis of the toxin-antitoxin reaction, it would appear that the latter is essentially a humoral process, which does not depend upon an active participation of the host. The animal, under such conditions, simply serves as a passive biological index of the amount of free toxin present, no implication of any phagocytic cells being primarily involved in this reaction. In harmony with the above presumption, it was found, when we inject *in vitro* prepared mixtures of tetanus toxin and antitoxin into blocked mice, that the ensuing reaction was in each instance directly comparable and alike with the corresponding effect in normal animals. Essentially different results, however, were obtained, when the antitoxin was given prophylactically and the degree of passive immunity tested against subsequent intoxication. Under such conditions, it was observed that the passive immunity conferred after an interval of 24 and 48, hours respectively, was much lower in blocked mice than in normal controls, a still further reduction becoming manifest after a 72 hour period. In interpreting this observation it is not clear whether the union of toxin and antitoxin *in vivo* depends upon a preceding fixation or attachment of the antitoxin to the reticulo-endothelial or other tissue cells (*cf.* Levaditi and Mutermilch (27), Hahn and von Skramlik (28)) which would be influenced unfavourably by previous blockade, or whether the lower protection is to be attributed to a more rapid excretion in the blocked animals of the antitoxin, which in this case was intimately bound to the foreign protein (horse serum pseudoglobulin). The observations of Saxl and Donath (29) on the slower elimination from the blood stream of foreign substances in splenectomized or blocked animals, and those of Collon (30), which suggest a prolonged presence of foreign protein in the circulation of rabbits after blockade with trypan blue, may be taken to indicate an inhibiting or retarding influence of blockade on the resorption of the antitoxin by the tissue cells. In the case of passive immunity to diphtheria, Kassowitz (31) and Freud (32) have shown, in addition, that a high concentration of antitoxin in the circulation may under certain conditions be associated with a low content of the

antibody in the tissues, in which case the individual would not be protected. The fact, however, that with the increase of the time interval protection was further diminished in the blocked animals in the writer's experiments, would render an explanation based solely upon an altered distribution of the antitoxin between the tissues and the circulation somewhat questionable. Further insight into these phenomena will not be gained until more is known of the elimination of heterologous antigens and homologous antibodies from the circulation and of the mechanism underlying their final excretion from the body, particularly with reference to changes in permeability of the cell membrane following the injection of blocking substances.

## SUMMARY.

1. Blockade of the reticulo-endothelial system by means of one intravenous injection of India ink as well as splenectomy did not alter the course of either Pneumococcus Type I infection or tetanus intoxication in mice.

2. The protective action of Antipneumococcus Type I serum against the corresponding infection, as determined by the injection of *in vitro* prepared mixtures of serum and culture, was definitely lower in mice which had received one blocking injection of India ink shortly before the test.

3. Titration of tetanus toxin and antitoxin in blocked and splenectomized mice gave results identical with those obtained in normal mice, if *in vitro* prepared and incubated toxin-antitoxin mixtures were injected. The degree of protection, however, conferred by a preceding dose of antitoxin against subsequent intoxication, was markedly lower in blocked mice than in normal control animals, this difference becoming more pronounced with the increase of the time interval.

## BIBLIOGRAPHY.

1. Boerner-Patzelt, D., Gödel, A., and Standenath, F., Das Retikuloendothel, G. Thieme, Leipsic, 1925.
2. Gay, F. P., *Arch. Path. and Lab. Med.*, 1926, i, 590.
3. Bieling, R., Erzeugung der Antikörper, in Kolle, W., and Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, Berlin, and Vienna, 3rd edition, 1927, ii, 133.
4. Krumbhaar, E. B., *Physiol. Rev.*, 1926, vi, 160.

5. Jaffe, R. H., *Arch. Int. Med.*, 1927, iv, 45.
6. Sacks, B., *Physiol. Rev.*, 1926, vi, 504.
7. Jungeblut, C. W., *J. Exp. Med.*, 1927, xlvi, 609.
8. Tsuda, S., *Virchows Arch. path. Anat.*, 1924, ccl, 136.
9. Hoen, E., Tschertkow, L., and Zipp, W., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 624.
10. Ledingham, J. C. G., *Brit. J. Exp. Path.*, 1927, viii, 12.
11. Wright, H. D. J., *J. Path. and Bact.*, 1927, xxx, 185.
12. Singer, E., and Adler, H., *Z. Immunitätsforsch.*, 1924, xli, 71, 468.
13. Oerskov, J., *Compt. rend. Soc. biol.*, 1925, xciii, 959.
14. Bass, F., *Z. Immunitätsforsch.*, 1925, xliii, 269.
15. Jungeblut, C. W., *Z. Hyg. u. Infectionskrankh.*, 1927, cvii, 357.
16. Kritschewsky, I. L., and Rubinstein, P. L., *Z. Immunitätsforsch.*, 1927, li, 27.
17. Lisgunova, A. W., and Butjagina, A. P., *Z. Immunitätsforsch.*, 1927, li, 56.
18. Feldt, A., and Schott, A., *Z. Hyg. u. Infectionskrankh.*, 1927, cvii, 453.
19. Bruynoghe, R., and Collon, N., *Compt. rend. Soc. biol.*, 1927, xcvi, 213.
20. Mutermilch, S., *Ann. Inst. Pasteur*, 1911, xxv, 776.
21. Neufeld, F., and Meyer, H., *Z. Hyg. u. Infectionskrankh.*, 1925, ciii, 595.
22. Meyer, H., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 124.
23. Lippmann, *Z. Immunitätsforsch., Orig.*, 1915–16, xxiv, 107.
24. Kritschewsky, I. S., and Meersohn, I. S., *Z. Immunitätsforsch.*, 1926, xlvii, 407.
25. Kolpikow, N. W., *Z. Immunitätsforsch.*, 1926, xlviii, 182.
26. Rosenthal, F., and Spitzer, F., *Z. Immunitätsforsch.*, 1924, xl, 529.
27. Levaditi, C., and Mutermilch, S., *Compt. rend. Soc. biol.*, 1913, lxxiv, 614.
28. Hahn, M., and von Skramlik, E., *Biochem. Z.*, 1922, cxxx, 80; cxxxi, 315.
29. Saxl, P., and Donath, F., *Wien. klin. Woch.*, 1924, xxxvii, 635; 1924, xxxviii, 66.
30. Collon, N. G., *Compt. rend. Soc. biol.*, 1927, xcvi, 427.
31. Kassowitz, K., *Z. ges. exp. Med.*, 1924, xli, 160.
32. Freud, P., *Z. ges. exp. Med.*, 1924, xlii, 400.

# THE EXTENT OF THE CAPILLARY BED OF THE HEART.

By JOSEPH T. WEARN, M.D.

With the Technical Assistance of LOUISE J. ZSCHIESCHE.

(*From the Thorndike Memorial Laboratory of the Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.*)

Plates 13 to 16.

(Received for publication, September 9, 1927.)

This investigation was begun with the object of determining the relative number of capillaries in normal human hearts as contrasted with the number in abnormal hearts from patients who had shown clinical signs of cardiac failure. In order to approach the problem it was first necessary to establish the size of the capillary bed and the distribution of the capillaries in normal hearts. The present paper, therefore, deals with the capillaries of the normal myocardium as determined by injections through the coronary arteries in man, cats and rabbits, and with the methods used in making these injections.

The coronary vessels have held the attention of numerous investigators for the past 3 centuries. Earlier workers studied their distribution by careful tedious dissections; and during the last few decades the vessels have been injected and outlined with various dyes and masses which have made possible a detailed study of the finer branches. Unfortunately, however, the injection masses used in most instances have failed to penetrate the capillaries and, as a result, the existing knowledge of this important group of vessels is very meagre. The same could be said, moreover, of the capillaries elsewhere in the body until the recent studies of Dale and Laidlaw (1) and Dale and Richards (2) on the effect of histamine upon the circulation, demonstrated the enormous capacity of the capillary bed. Krogh (3) subsequently directed attention to the number and behavior of capillaries in skeletal muscle, and, by his brillant investigations, opened a new pathway to this extensive but obscure branch of the vascular tree. In 1919, Richards (4, 5) made direct observations upon the kidney of a living frog and was able to study the behavior of the capillaries in that organ.

The heart does not permit of direct observation of the circulation in its walls, although Drury and Smith (6) have observed the blood flow in the branch of the coronary artery which supplies the first part of the pulmonary artery in the

turtle. Attempts to inject the capillaries of the heart have met with indifferent and not altogether uniform results. No effort has been made to determine the relative number of capillaries in the heart and very little study has been directed to their relationship to the muscle fibres, their anastomoses with one another or to their connections with the Thebesian circulation. One worker only, A. V. Meigs (7), injected two hearts with Berlin blue and his study led him to the conclusions that the capillaries in the heart were much larger than those elsewhere in the body and were unique in that they actually pierced and entered the cardiac muscle fibres. The sections of heart muscle reproduced in his paper, however, show an incomplete injection of the capillaries, and his evidence is not wholly convincing. Many other workers have failed to fill the capillaries and Nussbaum (8) was led to say that complete injection of the capillaries of the heart was impossible.

*Methods.*

Several methods for injecting the capillaries have been employed in the course of the present work, some of which have been successful in a few instances only, while one method in particular has been satisfactory in that it has given a uniformly and constantly complete injection of the capillary bed. Both the successful and unsuccessful methods will be described, the former in some detail, inasmuch as many of the experiments which were regarded as unsuccessful and resulted in incomplete injections of capillaries later proved to be of considerable value in a study of the Thebesian vessels.

In selecting materials for the injection it was necessary to choose substances that were soluble or, if in suspension, with particles of such diameter that they would enter the capillaries without difficulty. The other requirements for the injection material were that it remain in the capillaries, show no extravasation and withstand the process of fixation, embedding and staining. Berlin blue and India ink[1] have been employed frequently for injecting blood vessels and in this investigation they proved to be the most satisfactory of the various substances in suspension. Solutions of diazine green and trypan blue were used in some experiments with success, and many other dyes were tried but with disappointing results.

The first observations were made in hearts removed from cats after death and the method of making the injections was that which

[1] Either Weber's or Higgins' India ink was used.

had been commonly employed by most investigators in studying the coronary circulation.

The hearts were kept in the ice box for 48 hours post mortem in order to allow rigor to pass off. In preparation for injection they were perfused with 0.85 per cent salt solution until the perfusate came through practically clear and the heart appeared to be free from blood. This perfusion was carried out at various pressures ranging from 40 to 250 mm. Hg and at a temperature of 45°C. In some of the experiments the temperature was kept constant at 37°C. After washing out the blood with the perfusing fluid, the injection was made at the same temperature and pressure. A 2 per cent suspension of Berlin blue or India ink diluted with an equal part of distilled water was found to be the most satisfactory injection material. Both the saline solution and the injection suspensions were filtered before using in all experiments to be described hereafter. It was soon found, however, that this method, even when most carefully controlled, failed to inject all the capillaries. When the method was applied to human hearts, it proved even less efficient, and in many instances very few of the capillaries were filled. This failure was due in part to the escape of the dye through the Thebesian vessels.[2]

The same procedure was carried out on hearts obtained immediately after death with results slightly more encouraging but far from satisfactory.

In some of these experiments potassium sulfocyanate (3 per cent) was perfused through the coronary arteries to prevent constriction of the vessels, but this addition contributed little or nothing to the success of the method. The results were not dependable, for in some hearts scarcely a dozen capillaries per low power field were injected, while in others there were areas where many of the capillaries were filled.

After many other methods had been tried, partial success was obtained by omitting the washing out of the vessels, and injecting the dye without this preparation. Kerr and Mettier (9) found this procedure satisfactory in their study of the vascularity of the heart valves. The results obtained with this method, though better than the first, were not successful in that all the capillaries of the myocardium were not filled. A possible explanation of the variation in the results of this method was furnished in a series of experiments dealing with the Thebesian vessels. In brief, it was found that India ink, injected into the coronary arteries, escaped for the most part (60 to 90 per cent) through the Thebesian vessels and the capillaries remained unfilled. If, on the other hand, there was scant flow from the Thebesian veins and most of the ink escaped through the coronary sinus

[2] The part played by the Thebesian vessels is included in another paper in this issue.

and veins, the capillaries were apt to be completely or almost completely filled. This method, therefore, in some instances, gave beautiful injections, but it was not dependable and for this reason it was not adopted as the method of choice (see Figs. 1 and 2).

Attention was next directed to living hearts, and the first series of experiments was performed upon cats and rabbits anesthetized with sodium barbital. They were carried out in the following manner.

After complete anesthesia had been obtained, a tracheotomy was done and artificial respiration instituted. The lower part of the sternum was removed with enough of the ribs to expose the entire pericardial sac. Careful hemostasis was practiced and the structures handled as little as possible. Finally, after opening the pericardium in such manner as to bring the left auricle and ventricle into view, the dye was injected through a needle into the left auricular appendage, at a pressure very slightly in excess of that within the auricle. India ink or a dye was injected until the heart showed complete injection grossly, and this after some practise was determined by the appearance of the heart. If ink were used, for instance, the heart became uniformly black only when the capillaries were filled. At this point of maximum filling of the vessels some substance, such as formalin or alcohol, was injected through the same needle in an effort to stop the heart.

The following protocol, which represents the only successful experiment by this procedure, is illustrative.

*Experiment Cat 1.*—Weight of cat 3 kilos. May 14, 1924. Anesthesia was induced by means of sodium barbital (0.6 gm. per kilo intraperitoneally). Blood pressure was then registered by means of a cannula in the carotid artery. The chest was opened under artificial respiration and the pericardium incised in such a manner that the left auricle and ventricle came fully into view. India ink diluted with an equal part of distilled water and at a temperature of 37°C. was then injected directly into the left ventricle. As the injection began the aorta was clamped at the level of the right carotid artery. The pressure used in injecting the ink was barely sufficient to cause the ink to enter the ventricle against ventricular pressure. When the heart began to turn black, showing a capillary injection, 5 mg. of histamine was injected with the ink. The total amount of ink used was 20 cc. and the injection was made at such a rate that the blood pressure never rose above the original level.

At a certain time during the injection the heart turned very black and at this point a large clamp was placed on the heart in the auriculoventricular groove. The vessels at the base were also clamped, the heart excised and placed in 10 per cent formalin with the clamps in place. This heart ceased to beat almost immediately after the clamp was placed in the auriculoventricular groove. The injection grossly appeared to be perfect.

The experiment reported, however, together with another similar one, Fig. 3, furnishes the only two completely injected hearts obtained thus far in intact animals by injections made directly into the heart with Berlin blue and India ink. The obvious reason why these were successful is that the heart was stopped at the time of the maximum injection of the capillaries. In every other experiment attempts to bring about instantaneous stoppage were unsuccessful[3] and as a result the heart, by continuing to beat, "milked out" the dye from its capillaries. This phenomenon held true when the injected material was a suspension such as India ink or Berlin blue, for in many instances small particles of dye or ink adhered to the capillary walls and outlined them very faintly.

The method which finally gave the most satisfactory and almost uniformly successful injections was found to be the perfusion of the isolated beating heart. The method in brief was carried out in the following manner.

Rabbits or cats were killed instantly by a blow on the head. The heart was excised and a cannula tied into the aorta at a sufficient distance from the aortic valves to prevent blocking of the openings of the coronary arteries. The perfusion was immediately started at a pressure of 45 cm. of water, the temperature being kept at 37°C. The perfusate used was oxygenated Locke-Rosenheim solution (10). The chemicals used in the solution were the purest to be obtained upon the market. The water used was distilled from sodium hydrate, the entire distilling apparatus being made of Pyrex glass. All cannulæ and apparatus employed in the experiment were thoroughly cleaned and finally rinsed with the glass-distilled water before using.

With some practise a heart can be excised and the perfusion started before the heart ceases to beat, but even though it stops beating it is well known that it will begin to beat almost immediately after the perfusion is under way. The method as used will be recognized as a slight modification of the old Langendorff method.

The rate and strength of beat were measured by means of a spring lever tied to the tip of the apex. The coronary flow was collected and measured in a tipping bucket which recorded given amounts upon the smoked drum of the kymograph. When the experiment thus described was properly carried out, the rate of beat and coronary flow soon became constant with a liberal flow through the coronary

[3] Injection of chloroform, digitoxin and very strong currents applied to the heart stopped it in violent systole and this pressed the dye or ink from the capillaries.

vessels. In the event of faulty solution the coronary flow was very scant and the heart soon ceased to beat. When the constant flow level had been reached, a suspension of Berlin blue, 2 per cent, was injected into the stream near the opening of the coronary arteries. If the injection was successful the heart became almost instantly a dark blue color, and at this point it was stopped by the injection of 5 to 10 cc. of glacial acetic acid or formalin (10 per cent) in alcohol (95 per cent) into the perfusion fluid.

*Experiment Cat 3.*—February 12, 1926. The cat was killed by a sudden blow upon the head, and the heart immediately excised. A cannula was tied into the aorta and perfusion with Locke-Rosenheim solution immediately started. The perfusion pressure was 45 cm. of water and temperature of the perfusing fluid as it reached the heart was 37°C. The Locke-Rosenheim solution was well oxygenated. Coronary flow was registered by means of the tipping bucket, and after the heart rate and flow became constant a suspension of 2 per cent Berlin blue at a temperature of 37°C. was injected into the perfusing fluid near the coronary arteries, and the heart immediately became a deep blue, showing complete capillary injection. This was followed by 10 per cent formalin in 95 per cent alcohol, which caused the heart to stop beating.

*Experiment Rabbit 4.*—July 23, 1926. The heart used in this experiment was obtained from a rabbit after a procedure similar to that described in Experiment Cat 3. A cannula was placed in the aorta and perfusion was started with oxygenated Locke-Rosenheim solution at a pressure of 45 cm. of water at 37°C. After the flow became constant and the rate had become constant, Berlin blue was injected, a 2 per cent suspension, into the perfusion fluid. This was followed by glacial acetic acid which stopped the heart immediately. The heart became blue with the exception of a small spot which seemed to have blocked vessels. It was immediately placed in 10 per cent formalin in 95 per cent alcohol for fixation (Figs. 4 and 12).

In view of the complete injection of the capillary bed obtained by this method in the hearts of animals, it was applied to human material obtained at necropsy. Hearts obtained within 3 or 4 hours post mortem began to contract regularly when perfused with oxygenated Locke-Rosenheim solution although in some instances the left ventricle remained inactive if rigor had set in.

*Experiment Heart 52.*—July 15, 1926. This heart was obtained 2 hours post mortem from a boy aged 15 who died of Hodgkin's disease. In the space of another 20 minutes perfusion was begun with oxygenated Locke-Rosenheim solution at 37°C. and 50 cm. of water pressure. In about 5 minutes after the perfusion was begun the auricles began to twitch, this was followed immediately by regular beats, which in turn were followed by ventricular beats. After 10 minutes or so had elapsed the heart was beating normally, ventricular beats following the auricular

beats in regular order. The rate of beat was 62 per minute. The beats gradually increased in strength and finally the left ventricle was spurting the solution through the aorta with good force. When the flow had become steady a 2 per cent suspension of Berlin blue was injected into the perfusing fluid at such speed as to replace the perfusing fluid.

The heart immediately became a deep blue, but the Berlin blue flow was maintained for about 3 minutes, during which time the heart beat gradually weakened but did not cease. Glacial acetic acid was then run into the perfusing fluid and this stopped the heart instantly.

The heart was then sectioned, each section being carefully measured, after which they were placed in aqueous formalin for fixation (Figs. 5, 6 and 7).

Following such injections, the hearts were sectioned, placed in 10 per cent aqueous formalin or 10 per cent formalin in 95 per cent alcohol, for fixation, and, finally, after the standard preparation, were embedded in celloidin and sectioned. If it was desired to count the number of capillaries per sq. mm., careful measurements were made of the blocks of tissue before and after fixation in order that corrections might be made for the shrinkage during fixation.[4]

Sections of the muscle were stained according to the study to be made. When being prepared for counting capillaries per muscle fibre, a van Gieson or lithium carmine stain (11) was employed. If counts of capillaries per sq. mm. were to be made, the muscle was stained with picric acid, or phloxine for contrast to the color of the injection material.

### *Method of Counting Capillaries.*

The number of capillaries in the heart muscle has been determined in two ways. The total number per sq. mm. of muscle has been counted, and the number of capillaries per 1000 muscle fibres has been counted. In counting the capillaries by either method sections were selected in which the fibres and capillaries were cut transversely. The actual counting was then carried out by placing a ruled micrometer field in the eyepiece of the microscope and making the counts of the capillaries and fibres by the same method that one uses in counting erythrocytes in the ordinary chamber.

In counting the muscle fibres that had been cut in cross-sections slight difficulties were encountered because of the branching structure of the fibres. Thus a fibre sectioned at the exact point of branching sometimes appeared so that it

[4] All figures in this paper have been corrected for shrinkage. The method for counting capillaries per sq. mm. is adopted in order to compare the number found in normal hearts with that found in pathological hearts. These studies are now in progress.

might have been interpreted either as one or two fibres. Here the personal equation entered in. No claim is made that the counting of the fibres was absolutely accurate, but rules of counting were adopted and followed throughout and the fact that different people were able to make practically duplicate counts was sufficient to show that this error was not a serious one.

In each heart, capillary counts were made from numerous places in the walls of the ventricles, the auricles, the septum and the papillary muscle. The final figures given in the tables represent the average of many counts, and in most instances, the average of the results of several counters. In almost every instance, a thousand or more capillaries were counted in a given area. The exceptions to this, of course, were in making the counts of the auricle, and of the Purkinje fibres where no single area could be found that contained as many as a thousand capillaries cut in cross-section. The counts were made in areas that appeared to be completely injected, and figures from areas where the injection was obviously incomplete are not included.

The results of counts are shown in Tables I to VII. Table I illustrates variations met with in the counts of capillaries from the same heart by different observers. Tables II to IV show the averages of all counts by all observers of the number of capillaries per 1000 muscle fibres. Tables V to VII show the averages of all counts by all observers of the number of capillaries per sq. mm. of heart muscle. The variations in numbers of capillaries in the normal heart of man, cat and rabbit were very slight—so slight indeed that all these hearts can be said to have approximately one capillary for each muscle fibre.

To say finally that these numbers represent a complete filling of every capillary is not possible, but the finding of the same number in various hearts of man, cats and rabbits is strong evidence in favor of a complete filling of all the capillaries. The even distribution moreover, favored total injection for in many instances when injections were incomplete the distribution of capillaries was uneven and the vacant spaces were easily recognized.

In all parts of the heart, with the exceptions of the auricular walls and the Purkinje system, the capillary supply was practically the same. In the auricles, however, the supply was less abundant and the number of capillaries per muscle fibre varied considerably (*cf.* Figs. 5 and 6). In the thickest part of the auricular wall, where the muscle fibres were large, the count approximated in many instances that of the ventricle, but the auricular muscle fibres were usually distinctly

smaller and of less diameter than those of the ventricle. In such areas the number of capillaries per muscle fibre, therefore, was less than in the ventricle. Moreover, in the very thin portion of the auricular wall the number of capillaries per muscle fibre was distinctly less.

TABLE I.

*Human Heart 52.*

| By whom counted | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle | Purkinje fibres | Muscle immediately adjoining Purkinje fibres |
|---|---|---|---|---|---|---|---|
| O. A. | 1000 | 1060 | 1010 | 1160 | | | |
| S. W. | 828 | 1196 | 993 | 924 | | | |
| J. T. W. | 1091 | 1133 | 1040 | 1079 | | 671 | 1040 |
| | 1108 | 1100 | 1018 | 1083 | | 476 | 1018 |
| | 1046 | | | 1058 | | 595 | |
| | | | | 1076 | | 607 | |
| | | | | | | 627 | |
| | | | | | | 631 | |
| L. Z. | 1004 | 1080 | 1090 | 990 | 511 | | |
| | 1050 | 1120 | 940 | 940 | 478 | | |
| | 930 | 1010 | 960 | 1009 | 459 | | |
| | 930 | 1040 | 930 | 980 | 484 | | |
| | 930 | 1030 | 960 | 960 | 461 | | |
| | | | | | 402 | | |
| A. C. E. | 1072 | 1054 | 1083 | 1104 | | | |
| | 1067 | 1069 | 1074 | 1101 | | | |
| | 1067 | 1072 | 1032 | 1077 | | | |
| | 1060 | 1094 | 1065 | 1061 | | | |
| Average of all counts | 1013 | 1081 | 1015 | 1040 | 466 | 601 | 1029 |

In comparing quantitatively the circulation of the Purkinje system to that of ordinary heart muscle, it must be remembered that the actual area of the Purkinje system is small and the number of fibres counted consequently was much less than the number of ordinary fibres counted.

TABLE II.

*Human Hearts. Average of All Counts.*

*Number of Capillaries Per 1000 Muscle Fibres.*

| Human No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle | Purkinje fibres | Muscle immediately adjoining Purkinje fibres |
|---|---|---|---|---|---|---|---|
| 40 | 1080 | 1030 | 950 | | 550 | | |
| | | 1120 | 1080 | | 520 | | |
| | | 1110 | 1110 | | 510 | | |
| | | 1110 | | | 570 | | |
| | | 1060 | | | | | |
| | | 1123 | | | | | |
| | | 1126 | | | | | |
| | | 1052 | | | | | |
| | | 1070 | | | | | |
| | | 1160 | | | | | |
| | | 1080 | | | | | |
| | | 1100 | | | | | |
| | | 1000 | | | | | |
| 52 | 1000 | 1060 | 1010 | 1160 | 511 | 671 | 1040 |
| | 828 | 1196 | 993 | 924 | 478 | 476 | 1018 |
| | 1091 | 1133 | 1040 | 1079 | 459 | 595 | |
| | 1108 | 1100 | 1018 | 1083 | 484 | 607 | |
| | 1046 | 1080 | 1090 | 1058 | 461 | 631 | |
| | 1004 | 1120 | 940 | 1076 | 402 | 627 | |
| | 1050 | 1010 | 960 | 990 | | | |
| | 930 | 1040 | 930 | 940 | | | |
| | 930 | 1030 | 960 | 1009 | | | |
| | 930 | 1054 | 1083 | 980 | | | |
| | 1072 | 1069 | 1074 | 960 | | | |
| | 1067 | 1072 | 1032 | 1104 | | | |
| | 1067 | 1094 | 1065 | 1101 | | | |
| | 1060 | | | 1077 | | | |
| | | | | 1061 | | | |
| 57 | 1058 | 965 | 1092 | 1090 | 608 | | |
| | 1170 | 985 | 1131 | 1030 | 567 | | |
| | 1214 | 971 | 1090 | 1080 | 541 | | |
| | 1177 | 941 | 1046 | 1080 | 505 | | |
| | 1183 | 940 | 1068 | 1080 | 441 | | |
| | 1165 | 978 | 1082 | 1084 | 460 | | |
| | 1070 | 946 | 1071 | 1108 | | | |
| | 1166 | 970 | 1038 | 1040 | | | |
| | 1060 | | 1034 | 1042 | | | |
| | 1120 | | 1109 | 1130 | | | |

TABLE II—*Concluded.*

| Human No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle | Purkinje fibres | Muscle immediately adjoining Purkinje fibres |
|---|---|---|---|---|---|---|---|
| 58 | 1024 | 1200 | 1236 | 1060 | 493 | | |
| | 1104 | 1080 | 1180 | 1080 | 546 | | |
| | 1060 | 1200 | 1143 | 1090 | 508 | | |
| | 1159 | 1146 | 1110 | 1050 | 513 | | |
| | 1135 | 1081 | 1095 | 1080 | 500 | | |
| | 1242 | 1133 | 1087 | 1069 | 482 | | |
| | 1085 | 1140 | 1099 | 1094 | | | |
| | 1095 | 1088 | 1083 | 1110 | | | |
| | 1233 | 1072 | 1139 | 1080 | | | |
| | 1145 | 1087 | 1127 | 1156 | | | |
| Average of all counts | 1075 | 1032 | 1071 | 1060 | 503 | 601 | 1029 |

The main branches of the Purkinje system lie immediately beneath the endocardium, and the sections in which the capillaries were quantitated were from the papillary muscles or from the septum. The Best stain for glycogen was first used to identify the conducting system but the hemotoxylin so obscured the blue dye in the capillaries that it was impossible to count them. Consequently the Best stain was modified by Miss Louise J. Zschiesche[5] so that when tissues had

[5] The method was carried out in the following manner:

1. Stain the sections lightly with alum-hematoxylin for 10 seconds or longer, enough to bring out the nuclei.

2. Remove and wash in several changes of water.

3. Stain for 15 to 20 minutes in the following solution as found in Mallory and Wright (Mallory, F. B., and Wright, J. H., Pathological technique, Philadelphia, 8th edition, 1924, 199).

| | |
|---|---|
| Stock carmine solution freshly filtered | 2 cc. |
| Household ammonia | 3 cc. |
| Methyl alcohol 95 per cent | 3 cc. |

Filter.

4. Differentiate for 5 to 7 minutes in the following solution, changing the fluid two or three times until it remains uncolored:

| | |
|---|---|
| Absolute alcohol | 80 cc. |
| Methyl alcohol 95 per cent | 40 cc. |
| Distilled water | 100 cc. |

been through the process the capillaries remained clearly visible. This stain showed the glycogen as sharply as the Best stain.

The capillary supply to the Purkinje system was found to be much less than that to the heart muscle and the supply decreased rapidly as the endocardium was approached (see Figs. 13 and 8). Thus the Purkinje fibres lying immediately beneath the endocardium had an average of about 600 capillaries per 1000 fibres, while the muscle fibres lying more centrally in a papillary muscle averaged in two counts, 1029 capillaries per 1000 fibres. This is shown beautifully in Fig. 13. In some instances very small clumps of Purkinje fibres, made up of ten or twelve fibres, had a blood supply equal to that of the muscle—one capillary per fibre—but this finding was an unusual one.

When the entire capillary bed was completely injected, the enormous wealth of the heart in capillaries came to light. Practically every heart fibre was in direct contact with one capillary while many were touched by two or more capillaries. The anastomoses between the capillaries were so numerous indeed that these branchings running across the parallel muscle fibres wove a tangled structure of muscle and blood vessels so complex that one fibre was often completely surrounded by capillaries. The longitudinal sections showed this interrelated mesh of fibres and capillaries very strikingly (Figs. 3 and 4).

When the tissue was fixed and embedded in such a way that the shrinkage caused the fibres to stand out separately it was clear that the capillaries lay between and did not actually pierce the muscle fibres, but their intimate entwining about the fibres and through the forks of branching heart fibres frequently gave the appearance of their actually entering the substance of a fibre.

---

5. Wash in several changes of 80 per cent alcohol.

6. Wash in one change of 95 per cent alcohol.

7. Stain about 30 seconds in 95 per cent alcohol to which a saturated solution of picric acid has been added (5 cc. picric acid solution; 50 cc. 95 per cent alcohol).

The length of time the sections are left in the picric acid and alcohol depends on the intensity of the color desired for the cytoplasm.

8. Wash in several changes of 95 per cent alcohol.

9. Oil origanum.

10. Balsam.

The long standing controversy over the occurrence of blood vessels in the heart valves has received renewed attention in the past few

TABLE III.

*Cat Hearts. Average of All Counts.*

*Number of Capillaries Per 1000 Muscle Fibres.*

| Cat No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle | Purkinje fibres |
|---|---|---|---|---|---|---|
| 3 | 1080 | 1048 | 1070 | 1043 | 701 | |
| | 1035 | 1085 | 1070 | 1029 | 490 | |
| | 1006 | 1068 | 1008 | 1024 | 458 | |
| | 1089 | 1072 | 1000 | 1027 | 463 | |
| | 1058 | 1035 | 1042 | 1056 | 486 | |
| | 1085 | 1102 | 1057 | 1030 | 650 | |
| | 1114 | 1046 | 1053 | 1033 | 558 | |
| | 1074 | 1058 | 1056 | 1044 | 509 | |
| | 1094 | 1050 | 1066 | 1046 | | |
| | 1129 | | 1051 | 1043 | | |
| 4 | 1050 | 1002 | 1100 | 897 | 370 | |
| | 976 | 1100 | 1037 | 970 | 490 | |
| | 1002 | 833 | 920 | 1008 | 423 | |
| | 989 | 979 | 942 | 990 | 402 | |
| | 1023 | 940 | 971 | 1000 | 455 | |
| | 1021 | 970 | 910 | 1061 | 358 | |
| | 1005 | 979 | 964 | 956 | 485 | |
| | 1016 | 981 | 1000 | 957 | 462 | |
| | 978 | 947 | 971 | 1014 | 454 | |
| | 971 | | | | | |
| 5 | 1071 | 1073 | 1079 | 1084 | 608 | 638 |
| | 1021 | 1087 | 1115 | 1110 | 567 | |
| | 1063 | 1071 | 1086 | 1102 | 492 | |
| | 1073 | 1048 | 1026 | 1096 | 686 | |
| | 1063 | 1051 | 1064 | 1018 | 374 | |
| | 1050 | 1048 | 1058 | 1125 | 405 | |
| | 1106 | 1076 | 1047 | 1048 | 456 | |
| | 1058 | 1069 | | | | |
| | 1061 | | | | | |
| Average of all counts | 1046 | 1031 | 1029 | 1031 | 491 | 638 |

years and several contributions have been made. Numerous methods have been employed to inject the vessels of the valves and the results

TABLE IV.

*Rabbit Hearts. Average of All Counts.*
*Number of Capillaries Per 1000 Muscle Fibres.*

| Rabbit No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle |
|---|---|---|---|---|---|
| 1 | 1140 | 1143 | 1118 | 1073 | 675 |
| | 1114 | 1131 | 1140 | 1084 | 655 |
| | 1070 | 1150 | 1030 | 1030 | 528 |
| | 1080 | 1200 | 1082 | 1030 | 521 |
| | 1030 | 1122 | 1100 | 1040 | 461 |
| | 1100 | 1040 | 1150 | 1030 | 542 |
| | 1140 | 1219 | 1102 | 1235 | 604 |
| | 1033 | 1117 | 1117 | 1096 | 574 |
| | 1121 | 1084 | 1122 | 1103 | 649 |
| | 1080 | 1091 | 1108 | 1104 | 623 |
| | 1081 | 1107 | | 1108 | |
| | 1029 | | | | |
| | 1126 | | | | |
| 2 | 1128 | 1103 | 1160 | 1107 | 634 |
| | 1020 | 1100 | 1099 | 1057 | 730 |
| | 1038 | 1100 | 1047 | 1087 | 666 |
| | 1086 | 1129 | 1038 | 1080 | 747 |
| | 1070 | 1060 | 1121 | 1017 | 581 |
| | 1094 | 1050 | 1118 | 1170 | 463 |
| | 1107 | 1070 | 1093 | 1093 | 761 |
| | 1070 | 1000 | 1070 | 1032 | 568 |
| | 1025 | 1010 | 1086 | 1047 | 747 |
| | 1097 | 990 | 1090 | 1000 | |
| | | 1128 | | | |
| 3 | 1027 | 1130 | 1049 | 1127 | 528 |
| | 1021 | 1084 | 1024 | 1220 | 604 |
| | 1066 | 1192 | 1048 | 1023 | 408 |
| | 1024 | 1081 | 1020 | 1044 | 429 |
| | 1021 | 1066 | 1017 | 1072 | 407 |
| | 1106 | 1020 | 1064 | 1008 | 403 |
| | 1021 | 1061 | 1046 | 1046 | |
| | 1106 | 1054 | 1075 | 1020 | |
| | 1021 | 1049 | 1068 | 1065 | |
| | 1069 | 1064 | 1120 | 1008 | |
| | 1035 | | | | |
| | 1036 | | | | |

TABLE IV—*Concluded.*

| Rabbit No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle |
|---|---|---|---|---|---|
| 4 | 1130 | 1093 | 1082 | 1150 | 666 |
| | 1098 | 1061 | 1077 | 1031 | 436 |
| | 1010 | 1009 | 1092 | 1028 | 385 |
| | 1070 | 1051 | 1038 | 1043 | 394 |
| | 1080 | 1050 | 1056 | 1063 | 463 |
| | 1041 | 1014 | 1070 | 1049 | 413 |
| | 1019 | 1029 | 1092 | 1024 | 511 |
| | 1028 | 1019 | 1060 | 1009 | |
| | | 1030 | 1031 | 1016 | |
| | | | 1100 | | |
| 5 | 1223 | 1039 | 1224 | 1135 | 597 |
| | 1100 | 1057 | 1037 | 1104 | 414 |
| | 1095 | 1064 | 1044 | 1050 | 506 |
| | 1122 | 1041 | 1044 | 1091 | 590 |
| | 1087 | 1030 | 1081 | 1090 | 511 |
| | 1047 | 1043 | 1053 | 1070 | 477 |
| | 1038 | 1020 | 1068 | 1008 | |
| | 1022 | 1035 | 1038 | 1022 | |
| | 1060 | 1019 | 1059 | 1046 | |
| | 1048 | 1054 | 1105 | 1041 | |
| Average of all counts | 1071 | 1072 | 1075 | 1044 | 520 |

TABLE V.

*Human Hearts. Average of All Counts.*

*Capillaries Per Sq. Mm.*

| Human No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Purkinje fibres | Muscle immediately adjoining Purkinje fibres |
|---|---|---|---|---|---|---|
| 52 | 5852 | 5330 | 4480 | 4600 | 2360 | 4920 |
| | 5674 | 5824 | 4427 | 4960 | 1800 | 5400 |
| | 5720 | 6023 | | 5600 | 1524 | |
| | 5808 | 5416 | | 4640 | 2176 | |
| | 5674 | 5590 | | 5464 | 2840 | |
| | 5632 | 5893 | | 5240 | 2880 | |
| | 5776 | | | 5600 | | |
| | | | | 5360 | | |
| | | | | 5600 | | |
| | | | | 4920 | | |
| | | | | 5400 | | |
| Average of all counts | 5734 | 5679 | 4453 | 5216 | 2263 | 5160 |

TABLE VI.

*Cat Hearts. Average of All Sq. Mm. Counts.*

| Cat No. | Left ventricle | Right ventricle | Septum | Papillary muscle | Auricle | Purkinje fibres | Muscle immediately adjoining Purkinje fibres |
|---|---|---|---|---|---|---|---|
| 5 | 3400 | 4668 | 4676 | 4800 | 3204 | 1802 | 4340 |
| | 5040 | 4015 | 3181 | 3240 | 3131 | | |
| | 3040 | 4316 | 3040 | 3080 | | | |
| | 4408 | 4216 | 4480 | 4436 | | | |
| | 4424 | 3857 | 4340 | 4340 | | | |
| | 4224 | 3997 | | | | | |
| Average of all counts | 4089 | 4178 | 3943 | 3979 | 3167 | 1802 | 4340 |

TABLE VII.

*Rabbit Hearts. Average of All Sq. Mm. Counts.*

| Rabbit No. | Left ventricle | Right ventricle | Septum | Papillary muscle |
|---|---|---|---|---|
| 3 | 6336 | 5676 | 4160 | 5413 |
| | 6292 | 5806 | 4096 | 5600 |
| | 6248 | 5763 | 4000 | 6393 |
| | 5896 | 5720 | 4000 | 5413 |
| | 5764 | 6196 | 4000 | 7000 |
| | 5896 | 6673 | 4064 | 5693 |
| | 5852 | 6933 | 4064 | 5413 |
| 4 | 5720 | 6504 | 7100 | 5600 |
| | 5490 | 6461 | 6480 | 6461 |
| | 5320 | 6030 | 6800 | 5240 |
| | 5120 | 5772 | 6100 | 6480 |
| | 5160 | 6076 | 6300 | 5480 |
| | 5400 | 5987 | 6600 | 5680 |
| | 4960 | 5815 | 7150 | 5400 |
| | | | | 5680 |
| | | | | 5960 |
| Average of all counts | 5674 | 6101 | 5351 | 5806 |

obtained have been very variable. No one has found the vessels constantly present in all valves and this is about the only point upon which all workers seem to agree.

Bayne-Jones (12), Kugel and Gross (13) and Kerr and Mettier (9) have helped to bring order from the mass of contradictory evidence, by demonstrating clearly the presence of vessels in many valves. The difference in the methods of injection used in the past probably accounts for the conflicting claims made. Kugel and Gross have used a method which injects only the finer arterioles as they were not interested in the capillaries. These workers have found the vessels to be inconstant but present in many valves. Kerr and Mettier, on the other hand, have made beautiful injections of the capillaries in the heart valves of pigs

Before any final verdict can be rendered upon the subject a sufficiently large number of hearts must be studied in order to establish the average occurrence. By injecting the beating heart it has been possible to demonstrate vessels in the auriculoventricular valves in many instances, and in the semilunar valves in a few instances, and the distribution of the vessels showed the injection to be complete (Fig. 9). Instead of ending blindly in the valve the capillaries could be seen to go down onto the leaflet of the valve, make an unbroken loop and return to anastomose with the vessels at the base of the valve. Similarly the vessels in the papillary muscles ran up to the chordæ tendineæ, looped over and returned toward the base of the muscle. In rare instances the vessels ran through the chordæ tendineæ to anastomose with the capillaries on the free portion of the valve (Fig. 10).

It was also true, on the other hand, that in other perfectly injected hearts in which the muscle capillaries and vessels at the bases of the valves were completely filled, the valves showed no vascularity. These valves when cleared showed beautifully injected intact capillary loops at their bases, but none of the capillaries ran down upon the valve proper.

In view of the fact that the first part of the aorta receives a blood supply from a branch of the coronary artery, it was not surprising to find its wall well filled with capillaries (Fig. 11). The parallel course of the arteries and veins in the aortic wall is very striking and characteristic of the vasa vasorum in the wall of this vessel. This rich blood supply in the general region where aortitis is most common may be of important clinical significance.

SUMMARY.

By means of injections made into the coronary arteries of beating hearts it has been possible to determine the number of capillaries in the normal heart muscle. This study has shown a very rich blood supply with an average of approximately one capillary for each muscle fibre in the ventricular walls and papillary muscles, and a less abundant supply in the auricular muscle and Purkinje system. The number of capillaries per sq. mm. of ventricular wall or papillary muscle is about twice that found by Krogh in skeletal muscle. Capillaries were not found constantly in the valves of hearts in which there was apparently a complete injection of the capillary bed. The method described for injecting the capillaries of the heart also provides a means of studying the blood supply to the muscle, valves and aortic wall in pathological hearts.

We wish to express our appreciation to Misses Sylvia Warren and Olivia Ames, and to Dr. A. C. Ernstene for their assistance in making many of the capillary counts shown in the tables.

BIBLIOGRAPHY.

1. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1918–19, lii, 355.
2. Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918–19, lii, 110.
3. Krogh, A., The anatomy and physiology of capillaries, New Haven, 1922.
4. Richards, A. N., *The Harvey Lectures*, 1920–21, 163; *Am. J. Med. Sc.*, 1922, clxiii, 1.
5. Richards, A. N., and Schmidt, C. F., *Am. J. Physiol.*, 1922, lix, 489; 1924, lxxi, 178.
6. Drury, A. N., and Smith, F. M., *Heart*, 1924, xi, 71.
7. Meigs, A. V., *J. Anat. and Physiol.*, 1899, xxxiii, 243.
8. Nussbaum, A., *Arch. mikr. Anat.*, 1912, lxxx, 450.
9. Kerr, W. J., and Mettier, S. R., *Am. Heart J.*, 1925–26, i, 96.
10. Locke, F. S., and Rosenheim, O., *J. Physiol.*, 1907–08, xxxvi, 205.
11. Mallory, F. B., and Wright, J. H., Pathological technique, Philadelphia, 8th edition, 1924, 98, 119, 199.
12. Bayne-Jones, S., *Am. J. Anat.*, 1917, xxi, 449.
13. Kugel, M. A., and Gross, L., *Am. Heart J.*, 1925–26, i, 304.

## EXPLANATION OF PLATES.

### PLATE 13.

FIG. 1. 500 diameters. Human Heart 57. Ventricle. Good injections of capillaries made 48 hours post mortem. The injection was made through the veins without previous washing out of the vessels.

FIG. 2. 500 diameters. Human Heart 57. Ventricle. An incompletely injected area from a point near that shown in Fig. 1.

FIG. 3. 700 diameters. Cat 2. Ventricle. Longitudinal section of capillaries showing numerous anastomoses.

FIG. 4. 500 diameters. Rabbit 4. Papillary muscle. Cross-section of capillaries showing a field similar to that in Fig. 12.

FIG. 5. 500 diameters. Human Heart 52. Shows irregular and less abundant circulation of the auricle. Compare with Fig. 6.

### PLATE 14.

FIG. 6. 500 diameters. Human Heart 52. Longitudinal section of ventricle showing several anastomoses of capillaries.

FIG. 7. 500 diameters. Human Heart 52. Ventricle showing cross-section of muscle fibres and capillaries.

FIG. 8. Approximately 150 diameters. Human Heart 52. Papillary muscle showing Purkinje fibres at the upper edge of the section. Note the poorer supply of capillaries in the Purkinje tissue when contrasted with the adjoining muscle.

### PLATE 15.

FIG. 9. Human Heart 37. Cleared mitral valve, aortic cusp, showing clumps of capillaries in the valve. Note that the capillaries do not end blindly, but anastomose by loops with others nearby.

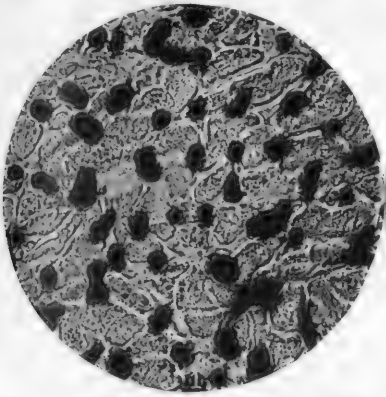
FIG. 10. Human Heart 37. Mitral valve showing anastomoses of vessels in chorda tendinea at the lower part of the plate with vessels on the free portion of the valve.

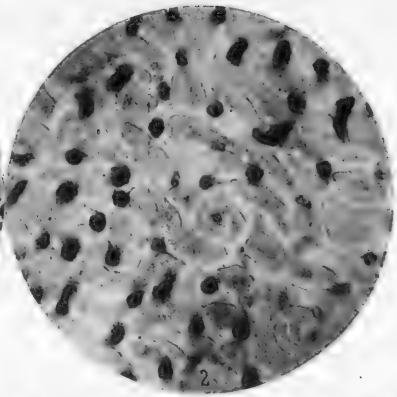
FIG. 11. Human Heart 52. Cleared section of aortic wall 1 cm. from the aortic valves.

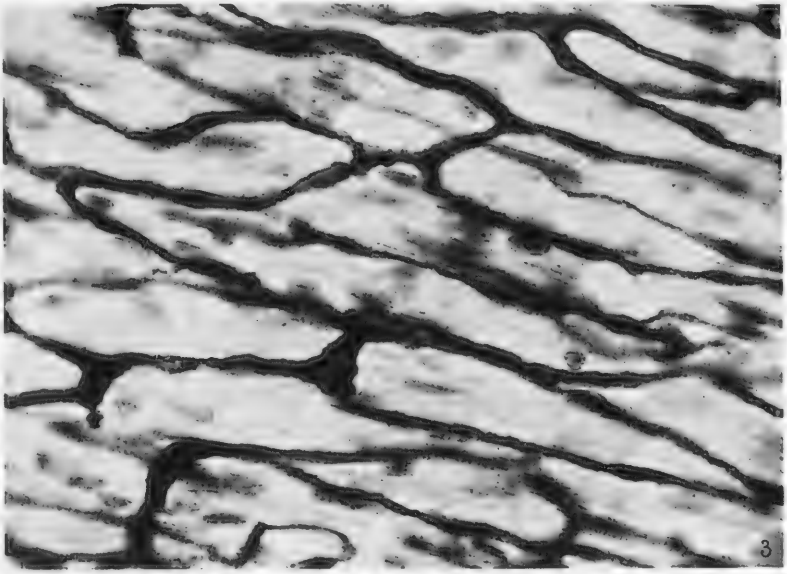
### PLATE 16.

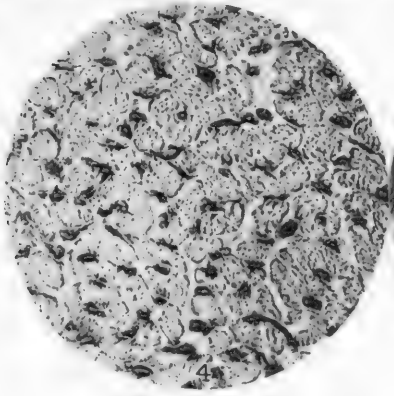
FIG. 12. Ventricle of Rabbit 4. Lithium carmine stain, showing red nuclei and red muscle fibres. The capillaries are filled with Berlin blue. This stain was employed to differentiate capillaries and cell nuclei, in the sections in which the capillaries were counted. This prevented confusion of nuclei and capillaries, and differentiated more clearly the single muscle fibre.

FIG. 13. Human Heart 52. Papillary muscle. Zschiesche modification of Best's stain. Capillaries are blue, Purkinje fibres are red and the ordinary heart muscle fibres are yellow. This plate shows much more clearly than Fig. 8 the difference in blood supply to the heart muscle and the Purkinje system.

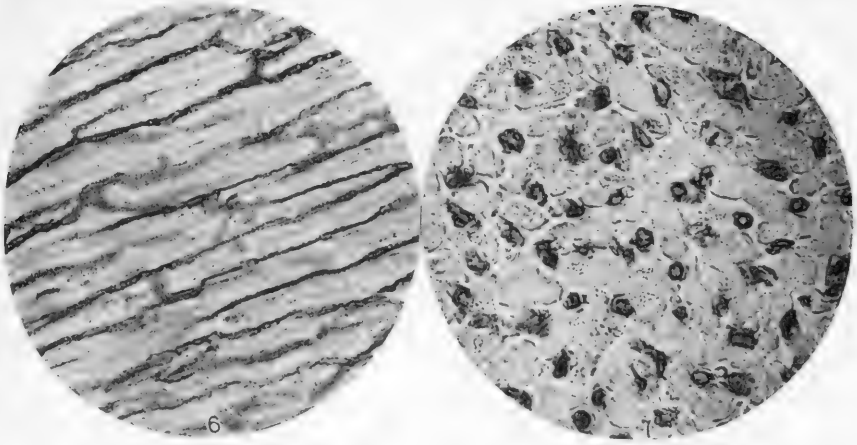




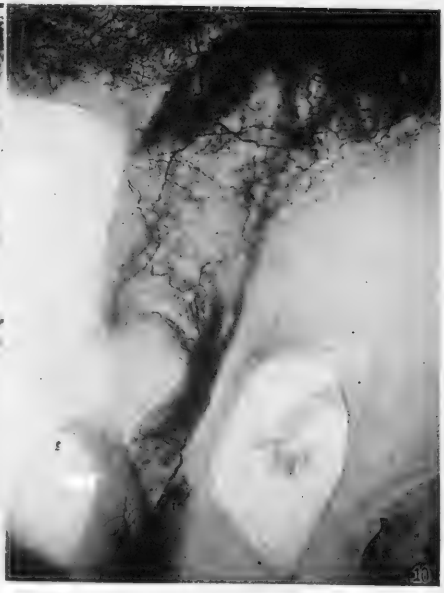




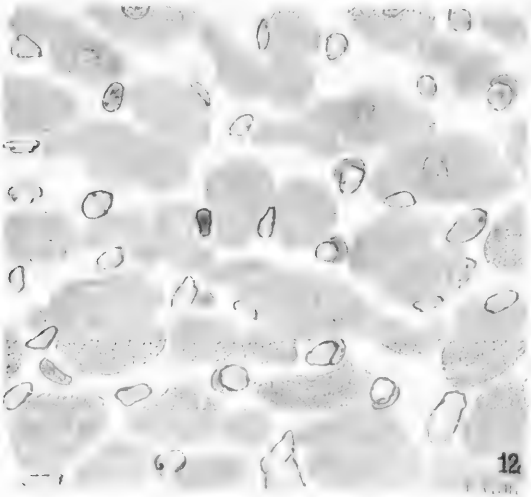
(Wearn: Capillary bed of heart.)



(Wearn: Capillary bed of heart.)



(Wearn: Capillary bed of heart.)



(Wearn: Capillary bed of heart.)

# THE RÔLE OF THE THEBESIAN VESSELS IN THE CIRCULATION OF THE HEART.

By JOSEPH T. WEARN, M.D.

*(From the Thorndike Memorial Laboratory of the Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.)*

Plates 17 and 18.

(Received for publication, September 9, 1927.)

Interest in the coronary circulation and blood vessels of the heart was very keen among the anatomists and physicians in the early part of the 18th century. They had begun to study the distribution of the coronary vessels and as a result of their dissections to construct theories of their function. Perfusion experiments were also carried out on human hearts obtained at necropsies and rapid advances were made in the knowledge of the coronary circulation. Text-books and anatomical works of the day devoted much space to the subject and clinicians began to correlate the postmortem with the clinical findings. The belief that special substances other than blood circulated in the blood vessels had begun to disappear.

This interest in the circulation of the heart walls was given a fresh stimulus by the publications of Raymond Vieussens in 1706 and by those of Adam Christian Thebesius 2 years later. Their discoveries of the channels connecting the coronary vessels directly with the chambers of the auricles and ventricles, introduced into the scheme of the circulation of the heart the vessels which are now generally known as Thebesian veins. In more recent times these vessels have received very little attention or study. Indeed, little is known of their anatomical relationships and no exact knowledge of the part they play in the coronary circulation exists. The importance, however, of a group of vessels which plays a part in the circulation of the heart itself, is obvious. It is the purpose of this paper to describe the previous work on the Thebesian vessels and their supposed functions and in addition, to present some observations made during the past 3 years

upon the relationship of these veins to the coronary arteries and upon some of their functions.

## LITERATURE.

In 1706 Raymond Vieussens (1) published the results of a series of experiments which he had carried out upon human hearts after death together with others on beef and sheep hearts. In these experiments Vieussens ligated the vena cava above and below the right auricle of the heart and next ligated the pulmonary veins. Having thus blocked these outlets he injected a solution of safranine in alcohol into the coronary arteries. After the safranine had filled the tissues of the heart, he observed that it ran out, not only through the coronary sinus into the right auricle, but—and this is the point of the experiment—it also escaped directly into the cavities of the heart by way of small ducts in the walls of the auricles and ventricles. These openings he termed "ducti carnosi." He found a considerable number of them on the interior surfaces of the heart and after an extensive study concluded that they were continuous with the coronary arteries.

In 1708 Thebesius (2) published his description of the numerous openings for the venous blood in the auricles and ventricles. He identified the openings by injecting the water into the coronary sinus and observing its escape into the chambers. The same results were obtained by injecting air into the coronary veins, and on still other occasions he employed colored liquids mixed with wax and glue. Thebesius raised the question as to why the Creator had placed these small veins in the walls of the heart, and then very obligingly answered it by saying that they made possible a continuous blood flow in the heart by serving as an exit at the beginning of systole. After thus answering the question so satisfactorily to himself, he gave due credit and much praise to the Creator for being so foresighted as to anticipate the need and usefulness of these little vessels. Thebesius also stated that while he was writing his paper he was shown the report of Vieussens' experiments. Despite the fact, therefore, that the vessels bear the name of Thebesius, it is to Raymond Vieussens that credit for their discovery should go. He reported his findings first, but of even greater importance was his method of injection, namely, through the arteries. Thebesius injected the veins and both men described the same openings.

Philip Verheyen (3) in 1712 reviewed the work of Vieussens and repeated some of his experiments. He conceded that the experiments were very unusual, but added that Vieussens, like other great men, had drawn too many conclusions from them. Verheyen confirmed the presence of the openings in the walls of the heart cavities, but explained them as openings of coronary veins and denied rather emphatically that they were special ducts. Neither his experiments nor his arguments in favor of his claims were very convincing.

In 1715 Vieussens published a book (4) in which he confirmed his former discovery of the common openings and spoke of their distribution and function. It was in this delightful publication that he described in detail stenosis of the mitral

valve and the clinical and necropsy findings in a patient with insufficiency of the aortic valve.

Lancisi (5) repeated the experiments and confirmed the results of Thebesius. His explanation of the raison d'être of the Thebesian system is interesting even if not based upon experiment. Assuming that the heart beat was started by the rush of blood into the heart muscle, he thought a rapid and simultaneous emptying necessary in all parts of the heart. In the parts most distant from the right auricle the necessary exits were supplied by the Thebesian vessels, and by this means, he said all parts could be emptied simultaneously and as a result all parts could be filled instantly.

Sénac is quoted (6) as saying that injections of air, mercury or tallow pass into the ventricles of the heart directly and enter these cavities equally well whether poured into the coronary artery or coronary vein. Langer (7), Bochdalek (8) and many others have admitted the presence of these vessels and studied their distribution in the walls of the four chambers. Nussbaum (9) also studied the numbers and sites of the openings but concluded that they existed only in the auricles.

On the other side of the question, however, are arrayed Cruveilhier (10) and Theile (11) who denied that such vessels existed. In reading their views one is led to believe that they are opinions only, and not based upon original observation. Lannelongue (12) too, denied that the small openings in the heart walls were connected with the arteries and veins. He maintained that they were merely continuations of the numerous small canals that ran behind the plexus of pillars in the walls of the ventricles.

Haller has been quoted by several writers as denying the existence of Thebesian vessels but in one of his writings (13) he spoke of their presence and stated that their function was to return the blood of those deep-seated coronary arteries which were unaccompanied by veins.

In more recent years Pratt (14) has demonstrated that an isolated cat heart can be kept beating for an hour by the perfusion of defibrinated blood through the Thebesian vessels. He believed the Thebesian veins were connected directly with the coronary veins and indirectly with the arteries through the capillaries. Pratt, moreover, explained the lack of infarcts in markedly sclerosed hearts by the presence of the Thebesian circulation.

The embryological development of the Thebesian veins has not been studied exhaustively but the few papers that exist indicate that these vessels are the remains of the primitive intertrabecular circulation of the embryo. Minot (15) found that the trabeculæ of the heart were made up in their earliest stage of young heart muscle cells covered with endothelium, but without capillaries of their own. It was his belief that the "sinusoidal" circulation of the embryonic heart was sufficient to nourish the trabecular muscle. Lewis (16) confirmed the work of Minot and followed the development of the sinusoids of the heart in the torpedo and rabbit and believed them to be the vessels that survive to some extent in the adult hearts, as the Thebesian veins.

Grant (17) has found that in the beginning the sinusoids were not connected with the coronary system, but grew into them at a later stage, as the coronary veins grew and branched in the myocardium. He (18) also described an anomaly of a child's heart in the ventricular muscle of which were large blood-filled spaces, communicating freely with the cavity of the ventricle as well as with the coronary vessels. They were interpreted as a persistence of the sinusoidal spaces of the embryonic heart.

In the hearts of certain fishes, turtles and frogs, the sinusoids persist in adult life, and it is through them that the inner surface of the heart wall is nourished. No capillaries have been found in the spongy surface supplied by the intertrabecular circulation.

## *Methods.*

The observations which stimulated the work to be reported upon in this paper, were made during attempts to inject the capillaries of a human heart. The method which was employed for the injection was as follows:

TABLE I.

*Human Heart 17.*

| | 1st measurement | 2nd measurement |
|---|---|---|
| | *cc.* | *cc.* |
| Outflow from pulmonary artery | 70 | 73 |
| Outflow from aorta | 190 | 190 |
| Outflow from coronary sinus and veins | 28 | 26 |
| Leaks from cut edges of heart | 60 | 52 |

About 48 hours after death a human heart was washed free of blood by perfusing salt solution (0.85 per cent) through the coronary arteries at a pressure of 160 mm. Hg. When the perfusate began to escape it was noted that the greater part of it ran out of the aorta and pulmonary artery and very little flowed from the coronary sinus. This observation was so startling that steps were taken immediately to measure the outflow from heart cavities and from the coronary sinus and other great veins. Glass cannulæ were introduced into the aorta and pulmonary artery in such manner that their ends extended well into the chambers of the ventricles. The coronary sinus and the posterior middle vein were also cannulated. At times several of the great veins emptied directly into the right auricle near the opening of the coronary sinus and just outside the valve of Vieussens. Whenever possible these veins were cannulated and the flow from them was

recorded. The coronary arteries were then perfused for 1 minute with normal salt solution at a pressure of 160 mm. Hg, and the perfusate was collected as it escaped from the various sources, and measured.

The results of such an experiment are shown in Table I.

Perfusion of salt solution (0.85 per cent) through the coronary arteries for 1 minute at 160 mm. Hg pressure.

Such results as those shown in Table I stimulated further study, and the experiment was repeated with the hearts obtained from a number of necropsies. Some of these were normal hearts, others were hypertrophied, while still others showed marked sclerosis of their vessels. The results of these experiments confirmed the findings in the earlier one (see Table II) namely, that 60–90 per cent of the coronary flow escaped through the Thebesian vessels directly into the chambers of the heart.

The possibility of rupture of some of the larger coronary vessels directly into the chambers or transudation of the salt solution into the cavities from the vessels was thought of as an explanation of the small amount of flow from the veins. Direct inspection, however, of the inner walls of the heart during perfusion with India ink showed the ink escaping only from the Thebesian vessels. Moreover, when the hearts were sectioned later, careful search failed to reveal any extravasation of the ink whatsoever. Further confirmation of the Thebesian vessels as the only source of the outflow from the chambers was furnished by perfusing with a suspension of gum acacia (6 per cent) in salt solution. This substance behaved in a manner similar to the saline perfusion, except that, as was to be expected, it ran more slowly (see Table II, Heart 43).

In several experiments agar and celloidin colored with dyes were perfused through the coronary arteries, and during the perfusion cold water or ice was introduced into the ventricles to harden these substances *in situ*. Subsequent sectioning of these hearts revealed small plugs of the injection mass protruding from the Thebesian openings. In one of the hearts in which celloidin was used as the injection mass seventeen openings of Thebesian vessels were found in the left ventricle with plugs of celloidin protruding from them (Fig. 1). The injection pressure used was 220 mm. of mercury and the mass was so thick

TABLE II.

| Human heart No. | Age of patient | Necropsy diagnosis | Weight of heart | Perfusate used | Flow from left ventricle (Thebesian veins) | Flow from right ventricle (Thebesian veins) | Flow (Thebesian veins) | Flow from coronary sinus and vein | Leaks from cut surfaces | Pressure of perfusate | Amount of flow | Condition of coronary arteries |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *yrs.* | | *gm.* | | *cc.* | *cc.* | *per cent* | *cc.* | *cc.* | *mm. Hg* | | |
| 15 | 45 | General peritonitis | 320 | Physiological salt solution .85 per cent | 88<br>79 | 122<br>120 | 42<br>44 | 105<br>100 | 175<br>151 | 190 | 490<br>450 | Normal |
| 16 | 45 | Chronic nephritis | 500 | Potassium ferrocyanide 3.2 per cent | 75<br>70 | 125<br>125 | 76<br>73 | 20<br>27 | 40<br>45 | 155 | 260<br>267 | Moderate arteriosclerosis |
| 17 | 50 | Acute purulent meningitis? | 410 | Physiological salt solution .85 per cent | 176<br>190<br>190 | 65<br>70<br>73 | 73<br>76<br>91 | 25<br>28<br>26 | <br>60<br>52 | 160 | 326<br>340<br>289 | " " |
| 20 | 50 | Sarcomatosis | 325 | " " | 235<br>240 | 250<br>260 | 78<br>74 | 50<br>80 | 80<br>95 | 220 | 615<br>675 | " " |
| 21 | 60 | Chronic myocarditis; heart failure | 300 | " " | 100<br>100 | 142<br>130 | 74<br>70 | 37<br>45 | 47<br>53 | 180 | 326<br>328 | Slight sclerosis of arteries |
| 22 | 51 | Chronic nephritis | 400 | " " | 175<br>175 | 235<br>215 | 74<br>74 | 20<br>15 | 120<br>120 | 150 | 550<br>525 | Moderate sclerosis |
| 24 | 42 | Bronchopneumonia | 220 | " " | 180<br>185 | 125<br>122 | 69<br>70 | 26<br>31 | 105<br>100 | 180 | 436<br>438 | Normal |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 50 | " | 260 | " " | 0 | 60 | 68 | 4 | 23 | 180 | 87 | Moderate arteriosclerosis |
| | | | | | 1 | 62 | 69 | 5 | 23 | | 91 | |
| 26 | 65 | Pulmonary tuberculosis | 500 | " " | 183 | 200 | 84 | 10 | 60 | 150 | 453 | " " |
| | | | | | 176 | 208 | 83 | 8 | 70 | | 462 | |
| 27 | *mos.* 19 | Bronchopneumonia | | " " | 19 | 32 | 34 | 16 | 80 | 150 | 147 | Normal |
| 37 | *yrs.* 40 | " | 250 | " " | 0 | 100 | 23 | 30 | 300 | 150 | 430 | Arteriosclerosis |
| | | | | | 0 | 100 | 23 | 33 | 300 | | 433 | |
| 43 | 43 | Pulmonary tuberculosis | 270 | " " | 140 | 215 | 72 | 60 | 75 | 150 | 490 | Normal |
| | | | | | 152 | 210 | 72 | 64 | 70 | | 496 | |
| | | | | Acacia 6 per cent | 175 | 175 | 58 | 75 | 170 | | 595 | |
| | | | | | 168 | 163 | 59 | 65 | 160 | | 556 | |
| 46 | 65 | Cerebral hemorrhage | 510 | Physiological salt solution .85 per cent | 232 | | 62 | 56 | 85 | 150 | 373 | Arteriosclerosis |
| | | | | | 246 | | 64 | 60 | 75 | | 381 | |
| 47 | 82 | Chronic nephritis myocarditis | 580 | " " | 210 | 300 | 76 | 35 | 125 | 150 | 670 | " |
| | | | | | 200 | 289 | 76 | 30 | 120 | | 639 | |

These experiments were carried out as nearly as possible under exactly the same conditions. All injections were made approximately 48 hours post mortem. The temperature of the perfusate was kept at 37°C. and with the exception of Heart 43 all of the outflows were collected during 1 minute periods. In Heart 43 the salt solution was perfused for 2 minutes and the acacia for 5 minutes.

that it did not enter the capillaries. Indeed, only the larger vessels were injected. No openings were found in the auricles and only two were found in the right ventricle. These experiments, therefore, confirm the previous ones (Table II) for here again the capillaries were not filled. Finally microscopic examination of sections from numerous parts of the hearts showed that the dyes and injection material had remained within the walls of the vessels.

Crainicianu (19), in perfusing hearts, noted this large escape from the ventricles but did not pursue it further. In 1913 Starling and Evans (20) during a study of the metabolism of the heart and lungs noted that about 40 per cent of the coronary blood did not return *via* the coronary sinus. They did not state, however, whether they took into consideration the other great veins which empty directly into the right auricle or the Thebesian flow to the left auricle and ventricle.

On gross inspection of the hearts used in the experiments of Table II it was obvious that the injection was uneven and incomplete. The larger vessels were well filled with ink or dye, but for the most part the heart was not colored by the injection material. Sections from numerous points in the walls of all the chambers were prepared and a careful microscopic study of these brought out the surprising fact that in most instances scarcely any of the capillaries were injected. The large arteries and veins were everywhere well filled or stained with the injection mass, but in the great majority of the microscopic fields very few if any capillaries could be found (Fig. 2). In certain fields small groups of capillaries were filled, but in no instance in the study of fifty human hearts was the capillary bed entirely injected. Even in the hearts in which some of the capillaries were injected they were so few in number that it was obviously impossible for them to take care of the profuse flow from the arteries into the cavities of the auricles and ventricles. In two hearts there was a scant flow through the Thebesian vessels, and in these several areas showed almost completely filled capillaries. The injection, however, was uneven and several large spots in the walls remained uninjected.

Here then is evidence that a direct connection exists between the coronary arteries and the chambers of the heart. The fact that so great a flow between the two can take place without going through the

capillaries shows the connecting channels to be of considerable size. The nature of this connection has not been definitely determined. It may possibly be a direct one from artery to Thebesian vessel, or more probably it may go from an artery into a large vein and thence into the heart cavity. Two of the smaller Thebesian vessels have been traced by serial sections, and their branchings studied. For convenience the smaller papillary muscles were selected because in so small a structure the course of the vessel would necessarily be a short one. In the two vessels so studied large venous branches were given off immediately beneath the endocardium and these branches divided immediately into venules and capillaries as illustrated in Fig. 3. Some of the Thebesian veins, therefore, drain the capillaries. This outlet for the capillary blood obviates the longer route of return through the veins to the coronary sinus and right auricle. The study has not progressed far enough to determine the relative number of Thebesian veins that drain capillaries as compared to those that connect directly with larger veins or through them with arteries. It is clear, however, that three types of connections exist, (1) the direct connection between the arteries and the Thebesian vessels, as shown by the celloidin injections, (2) the venous connection with the Thebesian vessels and (3) the capillaries which run directly into the Thebesian vessels.

The distribution of the Thebesian openings varied greatly but they were usually most numerous in the walls of the ventricles, in the pocket of the apex and in and around the bases of the papillary muscles. In the hearts studied there were relatively few openings into the auricles, and in two instances it was not possible to demonstrate any openings at all in either auricle. This was determined by measuring the flow from each chamber separately. The following protocol is illustrative.

*Protocol.*

*Heart 47.*—Male. Age 82. Chronic myocarditis with heart failure. A human heart, 24 hours post mortem, had cannulæ placed in the pulmonary artery, the coronary sinus and posterior middle vein, in the aorta and in the coronary arteries. The mitral valve was sewed together very tightly, as was also the tricuspid valve, in such fashion that they were water tight. A cannula was then sewed into each auricle. Following this, the coronary arteries were perfused at a pressure of 150

mm. of mercury, and flows from the various chambers were recorded for 1 minute periods. They were as follows:

| | 1st measurement | 2nd measurement |
|---|---|---|
| | *cc.* | *cc.* |
| Pulmonary artery | 300 | 289 |
| Aorta | 210 | 200 |
| Coronary sinus and vein | 35 | 30 |
| Right auricle | 0 | 0 |
| Left auricle | 0 | 0 |
| Leaks | 125 | 120 |

Following these measurements Berlin blue was injected into the coronary arteries at a pressure of 150 mm., and simultaneously carmine solution was injected through the coronary sinus and the posterior middle vein. The injection pressure continued for a few minutes following which the heart was opened and examined. It was interesting to note that most of the blue had emerged near the apex in each ventricle. The red was not so well localized. Neither red nor blue appeared in the auricles.

The number and size of Thebesian vessels in the various chambers also varied greatly. At times the flow was much greater from the right side while at others greater from the left, but the average from all the hearts showed a slightly greater flow from the right. The difference, however, was not significant.

Changing the perfusion pressure did not change the relative proportion of flow from the two sides of the heart, but did affect the rate of flow. Nor did the temperature of the heart influence the flow, for perfusions were carried out at various temperatures without effect upon the amount. It might be stated, however, that with few exceptions all the hearts were kept from 24–48 hours in the ice box, and then gently massaged and brought to a temperature of 45°C. before being perfused.

Attempts to inject the capillaries of a heart while in rigor mortis met with no better success. Nor did the use of this method with fresh warm hearts obtained soon after death give any better results. In still other hearts potassium sulfocyanate (10 per cent) was perfused through the vessels for 1 hour before they were injected with dye, as this procedure had been found to relax the greater vessels (21). These

hearts showed no better injections of the capillaries than did the others injected by the same method, in the absence of potassium sulfocyanate.

Vieussens, in his studies of the ducts opening into the heart chambers, made his injections into the coronary arteries, while Thebesius introduced his blowpipe into the coronary vein and observed the bubbles escaping into the immersed chambers. In this study the experiments of both Vieussens and Thebesius have been confirmed. Moreover, when perfusion was carried out through the coronary sinus at pressures ranging from 50–150 mm. Hg, almost all of the perfusate ran out through the Thebesian vessels, and in only a few instances did a few drops escape through the coronary arteries. This result was obtained when saline, acacia, agar and gelatin were used. Again in these hearts the capillaries were not injected, thus showing that the perfusate must have escaped by a more direct and larger communication between the veins and the Thebesian vessels, without having passed through the capillaries.

Perfusion was next carried out through the Thebesian vessels. Cannulæ were introduced into both coronary arteries, the coronary sinus and the other great veins which did not empty into the coronary sinus. All other outlets to the chambers were then carefully tied off after the chambers had been filled with 2 per cent Berlin blue in salt solution. Compressing the heart caused most of the fluid to run out of the veins, a few drops only escaping through the arteries. Histological sections of these hearts also showed little dye in the capillaries. The veins and a few arteries, however, were filled with dye.

These experiments have shown the existence of connections (1) between the arteries and Thebesian vessels and (2) between the veins and Thebesian vessels. In neither instance is the junction made through the capillaries alone. Before discussing the function of these channels it will be of interest to report the results of further experiments.

It will be noted that in all the experiments just described capillaries were rarely filled with the perfusing dye, and in no instance was more than a small fraction of the total number of capillaries injected. This held true even in the hearts where perfusion pressures were raised sufficiently to rupture the arteries.

It was routine practise in these experiments to wash the hearts free

of blood by perfusing them with saline before adding the dye to the perfusate. This procedure invariably distended the walls and dilated the chambers of the non-elastic dead heart. The mere perfusion distended it gradually and the fluid escaping from the Thebesian vessels stretched the walls as the cavities filled. Heyde (22) had shown that slight dilatation of the heart walls resulted in a slowing of capillary flow. These experiments confirmed that finding and failed to fill the capillaries while the heart walls were stretched.

In view of these findings the dye was introduced directly without previous washing out with salt solution in the manner described in the following experiment.

*Protocol.*

*Heart 44.*—This heart was obtained 2 hours post mortem and was still warm. Cannulæ were placed in the coronary arteries as quickly as possible and the heart, while the chambers were still collapsed and there was no stretching whatever, was injected with India ink diluted with an equal part of distilled water, heated to 45° at a pressure of 220 mm. of mercury.

The whole heart and aorta immediately became very black and the injection appeared excellent. Special care was taken to get the injection material in at a high pressure suddenly, to avoid distention of the walls of the heart. This is distinctly in contrast to the earlier human heart injections where the hearts were first perfused with salt solution in order to wash out the blood, and in which procedure the chambers were greatly distended. Later perfusion of hearts so injected with dye or ink invariably injected the Thebesian system and very few capillaries.

In this experiment and in all others carried out in the same manner a large number of the capillaries was injected, sometimes as many as 65 to 85 per cent of the total number. In several instances the injection was practically complete in certain areas of the muscle while in other parts the capillaries were only partially filled. It is of great import that the flow from the Thebesian vessels was very small in the hearts that showed such good capillary injection. In the earlier experiments where the coronary arteries were perfused with salt solution before the dye was introduced, it was noted that the capillaries were free from blood shortly after the perfusion was begun. The color of muscle changed from red to pale pink. Hence, when the dye or ink was injected without previous perfusion, it ran into the capillaries

until the heart became distended and then escaped through the Thebesian vessels into the cavities. This seemed the most plausible explanation of the results obtained. On this basis, therefore, the next step in the investigation was clearly indicated, namely, to study the flow from the Thebesian vessels in a beating heart. Consequently, human hearts obtained within 2 or 3 hours post mortem were perfused with oxygenated Locke-Rosenheim (23) solution. Usually they began to beat within a few minutes after the perfusion was started. The following experiment illustrates the method used.

*Heart 52.*—July 15, 1926. This heart was obtained 2 hours post mortem from the autopsy of boy, age 15, who died from Hodgkin's disease. Heart normal in size. Both coronary arteries were cannulated and a large cannula was tied into the coronary sinus. During this preparation the heart was kept at 37°C. in salt solution. 2 hours and 30 minutes postmortem perfusion of the coronary arteries was started with oxygenated Locke-Rosenheim solution. The temperature of the perfusion fluid was constant at 37°C. and the perfusion pressure was kept at 50–55 cm. of water. Within 5 minutes the auricles began to show very feeble beats and these were followed immediately by beats of both ventricles. A small dose of adrenalin (0.1 cc. of 1:1000) was injected very slowly into the perfusing fluid, and following this the auricles and ventricles began to beat strongly and normally, so that large spurts of fluid were forced from the aorta and the pulmonary artery. The rhythm became regular and the rate became constant at 62 beats per minute. The following flows were then recorded for 1 minute intervals.

After these flows were recorded a suspension of Berlin blue (2 per cent) in salt solution was injected into the perfusion cannulæ in the coronary arteries at sufficient rate to replace the Locke-Rosenheim solution. The pressure remained at 50 cm. water. The heart immediately turned to a deep blue color and glacial acetic acid was run into the coronary arteries. The acid stopped the heart beats instantly.

Similar results were obtained in another experiment, the single difference being that the left ventricle remained in systole and did not beat while the other three chambers were beating regularly. The outflow was measured during 1 minute before and after the heart began to beat.

Perfusion of the coronary arteries with Locke-Rosenheim solution at 50 cm. water pressure 37°C. 3 hours post mortem. The first flow was recorded before the heart began to beat. In this heart the left ventricle did not beat.

When the figures of Tables III and IV are contrasted with those in Tables I and II, the large outflow from the coronary sinus in Tables III and IV is very impressive, and all the more so when it is realized that the outflow from the other great veins not only was not included under the flow from the coronary sinus but was added to the ventricular outflow. In many of the experiments on dead hearts the outflow from the other great veins of the heart equalled that of the coronary sinus. The second column of figures, therefore, contains

TABLE III.

*Heart 52.*

| | From the coronary sinus | From the other veins, Thebesian vessels and leaks | Heart rate per min. |
|---|---|---|---|
| | *cc.* | *cc.* | |
| 1st measurement | 155 | 295 | 62 |
| 2nd measurement | 115 | 225 | 62 |

TABLE IV.

*Heart. 41. Male. Age 75. Death from Lobar Pneumonia. Heart Normal for Age.*

| | From the coronary sinus | From the other veins, Thebesian vessels and leaks | Heart rate per min. |
|---|---|---|---|
| | *cc.* | *cc.* | |
| 1st measurement | 205 | 360 | Not beating |
| 2nd measurement | 215 | 330 | 86 |
| 3rd measurement | 125 | 195 | 100 |

part of the venous outflow as well as the Thebesian flow and that part of the perfusate which escaped by leaking from the cut edges. This last source alone—the leaks—averaged much more in Table II than the flow from the coronary sinus and the veins. Of equal importance, too, in this heart was a fact brought out upon microscopic study of the sections from all parts of the muscle. The capillaries were completely and evenly injected (Fig. 4). Thus, in a beating heart, in the absence of dilatation, the capillaries were filled and the Thebesian flow was greatly diminished. The left ventricle which did not beat, was in

rigor and its capillaries were very imperfectly injected in contrast to those of the other chambers which were beating.

The results of these experiments were confirmed by another which differed only in the fact that flows were recorded before and after the heart began to beat. The protocol follows.

*Human Heart 53.*—Weight 300 gm. Male. Age 52. Death from pernicious anemia. This heart was obtained about 2½ hours post mortem. Cannulæ were introduced into both coronary arteries and into the coronary sinus, the latter for recording outflow. At about 3 hours postmortem perfusion with oxygenated Locke-Rosenheim solution was started through the coronary arteries. The temperature of the perfusion fluid was kept at 37°C. at the point of entrance into the arteries. The perfusion pressure was 55 cm. water. As the perfusion was started the chambers filled gradually and there was a very slight dilatation of the right ventricle, which increased gradually until the heart began to beat. Before any beats occurred the outflow was measured during two periods of 2 minutes each. They were as follows:

| | Outflow from coronary sinus | Outflow from other veins, Thebesian vessels and leaks |
|---|---|---|
| | *cc.* | *cc.* |
| 1st measurement | 210 | 525 |
| 2nd measurement | 190 | 510 |

After perfusing about 5 minutes 0.5 cc. adrenalin (1:10,000) was added very slowly to the perfusing fluid. Following this the right auricle began to twitch and immediately both auricles began to beat regularly. Next the right ventricle began to beat and finally the left ventricle joined in and beat very feebly. After another 5 minutes the rhythm was regular and the rate was constant at 78 per minute. At this point the flow was recorded for two more periods of 1 minute each, the results of which follow:

| | Outflow from coronary sinus | Outflow from other veins, Thebesian vessels and leaks | Rate per min. |
|---|---|---|---|
| | *cc.* | *cc.* | |
| 1st measurement | 260 | 550 | 78 |
| 2nd measurement | 268 | 545 | 78 |

Before further flows could be recorded the beat of the left ventricle became very weak and finally lost all force so that it merely twitched. At this point Berlin blue 2 per cent was introduced into the coronary artery cannulæ at such rate as

to replace the Locke-Rosenheim solution, and the beat of the heart immediately became weaker. Glacial acetic acid was then added to the perfusate and the heart beat ceased. Sections were cut from various parts of the walls, measured and placed in 10 per cent formalin (aqueous) for fixation.

The results of this experiment add to the value of the figures of the previous one (Heart 52) and in addition show a very slight increase of the venous outflow in the beating heart over the outflow before it began to beat. This heart also showed a good capillary injection except in the left ventricle which had stopped beating before the dye was injected.

The important point of this experiment is the relative change in the amount of outflow from the different sources when contrasted to the perfusion of dead hearts. In the beating heart (Heart 52) it did not

TABLE V.

*Heart 52 Beating. Heart 24 Not Beating.*

| | Outflow from Thebesian vessels* | Outflow from coronary sinus and veins | Leaks |
|---|---|---|---|
| | *cc.* | *cc.* | *cc.* |
| Heart 52 | 140 | 180 | 75 |
| Heart 24 | 305 | 28 | 102 |

* Average of combined outflow from the two ventricles.

seem wise to spend the time required in introducing cannulæ into the pulmonary artery, aorta and great veins emptying directly into the right auricle. Consequently, the outflow from the chambers was mixed with leakage from the cut surfaces, and in addition contained the outflow of all the veins emptying into the right auricle except that from the coronary sinus. In many of the perfusion experiments on dead hearts the outflows from the coronary sinus and from certain other great veins were measured separately and the average outflow from the latter was a third to a half of that from the coronary sinus. If one takes that figure as the flow from the other veins and assumes that the leakage was 75 cc. the following values can be calculated for the flows from the different sources.

When these calculations have been made by subtracting the leaks

and venous flow (other than from the coronary sinus) from collected outflow and adding the venous flow to that of the coronary sinus a figure is found which is quite in contrast to those found on perfusing dead hearts.

The evidence thus far considered has been obtained from hearts after death or from isolated beating hearts. In support of this evidence the results of thirty-five experiments on intact cats are offered. The cats were carefully anesthetized with sodium barbital and when completely under the anesthetic, the chest was opened under artificial respiration in such manner that the heart was exposed. A dye or India ink[1] was injected into the left auricle or left ventricle and the heart, by pumping the dye or India ink into the coronary arteries, injected itself. Immediately after the injection of India ink was started, the arteries first filled and appeared as black stripes on the heart. As the ink reached and filled the capillaries the entire heart became uniformly black but remained so only for a brief second or more when it suddenly began to dilate. During the dilatation, even though the ink injection was continuous, the beats became very feeble and the color changed from deep black to a pale pinkish color, as the feeble beats "milked" or squeezed the dye from the capillaries.

An explanation of the change in the color of the heart as it dilated and began to beat feebly was difficult, but it was furnished by the two experiments which follow.

*Cat 2.*—Weight of cat 3 kilos. May 14, 1924. Anesthesia was induced by means of sodium barbital (0.6 gm. per kilo intraperitoneally). Blood pressure was then registered by means of a cannula in the carotid artery. The chest was opened under artificial respiration and the pericardium incised in such a manner that the left auricle and ventricle came fully into view. India ink diluted with an equal part of distilled water at a temperature of 37°C. was then injected directly into the left ventricle. As the injection began the aorta was clamped at the level of the right carotid artery. The pressure used in injecting the ink was barely sufficient to cause the ink to enter the ventricle against ventricular pressure. When the heart began to turn black, showing a capillary injection, 5 mg. of histamine was injected along with the ink. The total amount of ink used was 20 cc. At a certain time during the injection the heart turned very black and at this

[1] Berlin blue 2 per cent suspension in normal saline. Either Weber's or Higgins' India ink was used, as a 50 per cent suspension in distilled water. Both dyes and inks were filtered immediately before using.

point the large clamp was placed on the heart in the auriculoventricular groove. The vessels at the base were also clamped, the heart excised and placed in 10 per cent formalin with the clamps in place.

This heart ceased to beat almost immediately after the clamp was placed in the auriculoventricular groove. The injection of the capillaries appeared to be perfect on gross examination.

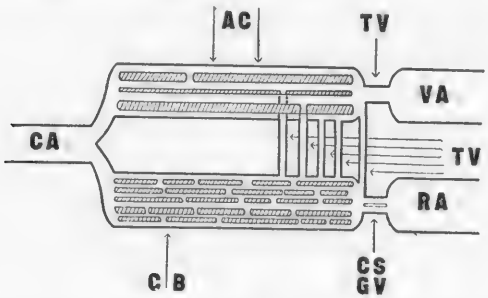
*Cat 5.*—Weight 3.1 kilos. May 20, 1924. Anesthesia was induced by means of sodium barbital (0.5 gm. per kilo intraperitoneally). Both carotid arteries were cannulated as was also the femoral vein. Blood pressure tracings were made from the left carotid and finally the abdomen was opened and a ligature placed around the aorta but it was not tied. Berlin blue (2 per cent suspension in 0.85 per cent salt solution) was slowly injected into the femoral vein. After approximately 10 cc. had been injected the blood pressure began to fall and the injection was made more rapidly from that time on, but the rate was never such as to raise the blood pressure above the original level.

The chest was hurriedly opened and it was found that the heart had stopped beating in systole and was deep blue in color. Additional Berlin blue was then injected through the right carotid artery after the ligature around the aorta had been tied. The injection pressure was 220 mm. of mercury. 10 per cent formalin was finally run in through the same artery at the same pressure. The chest was opened, the vessels of the heart clamped at the base, the heart excised and placed in 10 per cent formalin.

The important point that was common to these two experiments was the fact that the heart ceased to beat in each instance while the capillaries were filled and before it began to dilate. Furthermore, microscopic sections of both hearts proved the capillaries to be perfectly injected. The explanation to be given of this behavior, while not necessarily a final one, seems entirely reasonable. As the injection of ink was begun it entered the capillaries and cutting off the oxygen to the muscle, caused the heart to dilate. As dilatation occurred the capillaries were stretched and thereby further entrance of ink into them was prevented. But the feeble beats of the dilated heart were sufficient to squeeze the ink out of the capillaries. Such circulation as continued was almost certainly through the Thebesian vessels which were found well injected with the ink when the sections were studied with the microscope. Repeated efforts have been made to duplicate these experiments following each step with the greatest care, but all have failed because it has not been possible to stop the hearts at the proper stage during the injection, while the capillaries were filled. Sections in these later instances showed that only the large vessels were filled.

After witnessing these experiments one can hardly refrain from speculation as to the reason why the cardiac capillaries are filled with such difficulty and what part, if any, the Thebesian vessels play as an impediment to their filling. The results just reported furnish data which will serve as a basis for an hypothesis.

The facts that capillaries are perfectly filled only in a beating heart, that in these experiments at least, they fill less completely in a non-distended dead heart, and scarcely at all in a dilated heart, suggest the Thebesian vessels as a cause for the lack of success in the last two instances. The beating heart therefore must at some stage of its cycle close the Thebesian vessels and force the blood through the capillaries. This is not at all unreasonable when one considers the pressure relations in the left ventricle and the aorta during systole and diastole. If one accepts for the moment the presence of the direct communications between the




TEXT-FIG. 1. *C A* = coronary arteries. *A C* = arterial communications with Thebesian vessels. *T V* = Thebesian vessels. *V A* = cavities of ventricles and auricles. *C B* = capillary bed. *C S* = coronary sinus. *G V* = great veins emptying directly into the right auricle. *R A* = right auricle.

coronary arteries and the heart chambers on one hand and between the veins and chambers on the other, a chart can be drawn which illustrates diagrammatically the various vascular channels of the heart. The commonly accepted route of blood flow in the heart is from the aorta into the coronary arteries and thence through the capillaries into the large veins which empty into the right auricle directly or *via* the coronary sinus. In the diagram in Text-fig. 1, this route would be shown by having the blood flow from the coronary arteries (*C A*) through the capillary bed (*C B*), and the coronary sinus and veins (*C S* and *G V*) into the right auricle (*R A*). The experiments just reported have shown a direct communication between the coronary arteries (*C A*) and the heart chambers *via* the channels (*A C*) to the Thebesian vessels (*T V*). And in dilated dead hearts these channels

proved so readily accessible that approximately 60–90 per cent of the fluid perfused into the coronary arteries escaped through them. In beating hearts the amount was considerably less and more perfusate ran through the capillaries (*C B*) into the coronary sinus and veins (*C S* and *G V*). If the pressure that exists in life during the various phases of the cardiac cycle be considered, a hint of the use of the Thebesian vessels may be found. During ventricular systole the pressure is the same, of course, in the left ventricle and at the mouth of the coronary arteries, but the pressure in the right ventricle and in both auricles is less than it is in the coronary arteries. In diastole, however, with the aortic valves closed, the pressure in the coronary arteries (*C A*) is greater than in any of the heart chambers, hence an increased outflow from the Thebesian vessels (*T V*) into the heart chambers (*V A*) would be expected. Even during ventricular systole there is possibly a little outflow into the right ventricle, and an unhindered flow into the auricles. The number of openings in the auricles, however, is usually small.

This set of pressures would certainly retard the flow from the coronary arteries (*C A*) to the right ventricle and stop it from the coronary arteries (*C A*) to the left ventricle; and would, therefore, greatly inhibit the flow from the coronary arteries (*C A*) to the chambers (*V A*) during systole. The alternate route of flow would be through the capillary bed (*C B*). The Thebesian system, therefore, would serve as an additional means of escape of the blood in the heart wall and thereby enable the coronary vessels to empty themselves very rapidly. The veins also communicate directly with the Thebesian vessels, therefore the latter might serve as a means of rapid emptying of the entire vascular bed of the heart during diastole. Moreover, since some of the Thebesian vessels are connected directly with the capillaries, it may be possible for blood to flow from the ventricle through these vessels into the capillaries.

Such speculations as have been discussed are interesting but extremely difficult of proof. They are offered as possible explanations with full realization that further work is indicated which may or may not support them. So long as they are recognized as theory rather than fact they may serve to stimulate further study of the function of these vessels in normal hearts.

Finally another very important function of the Thebesian vessels has been demonstrated by the findings in two hearts at the postmortem table.[2] This function has been proved more conclusively by the clinical histories and necropsy findings in these two cases than it could be by any experiment in the laboratory. In each of the hearts

[2] It was possible to study and use these hearts through the kindness of Dr. Timothy Leary. The cases are being reported in detail in another paper.

there was complete closure of both orifices of the coronary arteries. The first one was found in a negro woman, about 26 years of age, who had worked and earned her living as a seamstress up to within a few days of her death. She was found dead in her room and as she lived alone the nature of her death could not be learned. A syphilitic process in the aorta, probably of long standing, had completely closed and obliterated the orifices of both coronary arteries.

The second heart was from a man 35 years old who at the time of his death was working as a seaman. It was learned that he had been considered very lazy by other members of the crew but he was able to do his work and so far as they knew, did it without any symptoms or signs of heart disease. Between his jobs as a seaman he worked for an awning company and assisted in putting up and removing awnings from houses. In this heart also there was a definite syphilitic aortitis which had closed the orifices of both coronary arteries.

Needless to say, in both instances a very careful search for other openings of the arteries was made, but without success. Attempts to inject fluid and pass probes through the orifices also failed. Moreover, sections through the vessels $\frac{1}{2}$ cm. outside the aorta revealed normal lumens. The orifices had, therefore, been closed by the disease in the aorta, and the closure almost certainly had been a very gradual one. Sections through the arteries at their orifices showed complete closure in all vessels and in these the process was of long standing.

In each case a heart without openings for the coronary arteries had maintained an efficient circulation which enabled the person to earn a living. The obvious questions are where and how did these hearts get sufficient circulation to function so efficiently? The answer is not difficult for the only other entrance to the coronary circulation is through the Thebesian vessels and in the instances just cited, at least, they were able to supply the heart muscle with the necessary blood to maintain a wage-earning life. It should be emphasized at this point that the closure of the coronary orifices in these two cases was a gradual one. It was this time element, most probably, that enabled the Thebesian circulation to take over the new duties and perform them so efficiently. One does not need to search far afield for evidence to support this claim. It is commonplace to find at necropsies extensive

sclerosis of the coronary arteries, so well advanced that it is difficult to introduce the point of a pin into the calcified lumens. In many instances these people had been comparatively well and healthy individuals, with dyspnea on exertion perhaps, but falling into the group of men considered normal at their age. In such conditions it is certain that the process of occlusion was a gradual one. Their clinical histories stand out in sharp contrast to those of patients with sudden closure of one branch of a coronary artery. Many of these die at the time of the closure while others recover after acute heart failure. In certain emergencies, therefore, particularly when allowed sufficient time to adapt themselves, the Thebesian vessels can take over the function of the coronary arteries.

## SUMMARY.

In summary, evidence has been presented to show a direct connection other than through the capillaries between the coronary arteries and the chambers of the heart.

This connection was shown by perfusion, injections and serial sections to be through the Thebesian veins. Communications between the larger coronary veins and the Thebesian veins were also demonstrated by the same methods.

Serial sections through Thebesian veins have shown capillaries draining directly into them. Under certain conditions it has been shown that as much as 90 per cent of the arterial flow may escape *via* the Thebesian vessels.

Lastly, in the event of gradual closure of the orifices of the coronary arteries, the Thebesian vessels can supply the heart muscle with sufficient blood to enable it to maintain an efficient circulation.

This work has been in progress for 4 years during which time Dr. William B. Stevens assisted in many of the earlier experiments. I wish to express my deep appreciation to Misses Olivia Ames and Sylvia Warren, and Drs. Henry Jackson, Jr., and Joseph M. Hayman, Jr., for their assistance in translating the older French and Latin papers; and to Dr. Francis W. Peabody for his very helpful criticisms and advice throughout the investigation.

## BIBLIOGRAPHY.


1. Vieussens, R., Nouvelle decouvertes sur le coeur, Toulouse, 1706.
2. Thebesius, A. C., Dissertatio medica de circulo sanguinis in corde, Lugduni Batavorum, 1708.
3. Verheyen, P., Anatomiæ corporis humani, Coloniæ, 1712.
4. Vieussens, R., Traité nouveau de la structure et des causes de mouvement naturel du coeur, Toulouse, 1715.
5. Lancisi, J. M., A secretiori cubiculo et Archiatri Pontificii, de motu cordis, et aneurysmatibus (opus posthumum), Lugduni Batavorum, 1740.
6. Sénac, J. B., Traité de la structure du coeur, Paris, 1749, quoted by Lannelongue (12).
7. Langer, L., Die foramina Thebesii in Herzen des Menschen, *Sitzungsber. k. Akad. Wissensch., Math.-naturw. Cl., Wien*, 1880, 3 Abt., lxxxii, 25.
8. Bochdalek, V. A., *Arch. Anat.*, 1868, 302.
9. Nussbaum, A., Über das Gefäszsystem des Herzens, *Arch. mikr. Anat.*, 1912, lxxx, 450.
10. Cruveilhier, J., Anatomie descriptive, Paris, 1834, iii, 1.
11. Theile, F. G., Myologie et angéiologie, in Encyclopédie anatomique, 1843, iii, 586.
12. Lannelongue, O., Circulation veineuse des parois auriculaires du coeur, Paris, 1867.
13. Haller, A., First lines of physiology, English translation, Edinburgh, 1786, i, 75.
14. Pratt, F. H., *Am. J. Physiol.*, 1898, i, 86.
15. Minot, C. S., *Proc. Boston Soc. Natural Hist.*, 1900, xxix, 185.
16. Lewis, F. T., *Anat. Anz.*, 1904, xxv, 261.
17. Grant, R. T., *Heart*, 1926, xiii, 261.
18. Grant, R. T., *Heart*, 1926, xiii, 273.
19. Crainicianu, A., *Virchows Arch. path. Anat.*, 1922, ccxxxviii, 1.
20. Evans, C. L., and Starling, E. H., *J. Physiol.*, 1913, xlvi, 413.
21. McCordick, quoted by Gross, L., The blood supply to the Heart, New York, 1921.
22. Heyde, I. M., *Am. J. Physiol.*, 1898, i, 215.
23. Locke, F. S., and Rosenheim, O., *J. Physiol.*, 1907–08, xxxvi, 205.


## EXPLANATION OF PLATES.

### PLATE 17.

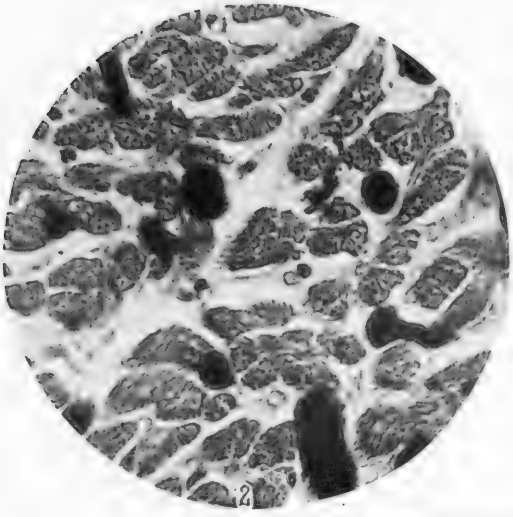
FIG. 1. Left ventricle of heart injected with celloidin, showing the celloidin plugs protruding from the Thebesian vessels. The plugs have been covered with white ink in order to make them show more distinctly in the photograph.

FIG. 2. Cross-section of muscle fibres from the left ventricle of Human Heart 14 showing the larger vessels well filled, but scarcely any capillary injection.

PLATE 18.

FIG. 3. One of a series of sections through a Thebesian vein (the channel running across the middle of the field) showing a vein and capillaries emptying into the Thebesian vein. This section lay less than 0.5 mm. below the endocardial surface.

FIG. 4. Cross-section of muscle fibres and capillaries of right ventricle of Human Heart 41 injected while beating.



(Wearn: Thebesian vessels in heart circulation.)

(Wearn: Thebesian vessels in heart circulation.)

# A STUDY OF THE MECHANISM OF RECOVERY FROM EXPERIMENTAL PNEUMOCOCCUS INFECTION.

By OSWALD H. ROBERTSON, M.D., SHUTAI T. WOO, M.D., SHEO NAN CHEER, M.D., AND LI PIN KING, M.D.

(*From the Department of Medicine, Peking Union Medical College, Peking, China.*)

(Received for publication, November 19, 1927.)

In previous investigations of the nature of natural immunity to pneumococcus infection (1–3) evidence of a defense process common to both pneumococcus-resistant and susceptible animals was found. It was possible to demonstrate, by appropriate methods, pneumococcidal activity in varying degrees with serum-leucocyte mixtures of the different animal species studied. The relatively marked pneumococcus-killing power of the naturally immune animals' blood was found to depend on the presence of opsonins capable of causing the phagocytosis and destruction of highly virulent pneumococci. The blood of susceptible animals lacked this property of influencing virulent strains but exhibited opsonic activity and destroying power for pneumococci of low virulence for the species. In relation to the total resisting capacity of the body, the degree of potential pneumococcus-killing energy resident in the blood suggests that it is an important if not the principal means of normal antipneumococcus defense. The question then arises as to whether recovery from pneumococcus infection is brought about by a similar mechanism or whether other and quite different forces are employed by the body in terminating the activity of pneumococci which have succeeded in overcoming the initial resistance of the animal. The results of a study of this problem by the same methods employed in the previous work constitute the subject of the present communication.

A review of the recent literature on immunity factors in recovery from experimental pneumococcus infection reveals several points of view which have arisen as a result of a diversity of experimental findings. Certain investigators, Bull (4), Tchistovitch (5), Tudoranu (6), and others, believe that recovery is

brought about by the elaboration of specific immune substances, agglutinins, opsonins, or protective bodies which promote the destruction of the disease producing microorganisms by the leucocytes. Other workers take an entirely opposite view, namely that recovery is quite independent of these manifestations of humoral immunity. Singer and Adler (7) eliminate humoral immunity completely from the mechanism and hold that a specific immune change occurs in the cells of the reticulo-endothelial system by which they are enabled to engulf and destroy the invading pneumococci. Wadsworth (8) considers that the production of humoral immune substances is an important element in the recovery process but is of the opinion that pneumococcus immunity is chiefly antitoxic in nature. The majority of investigators, however, have come to the conclusion that the elaboration of antibodies of the bacteriotropic or protective type constitutes a part of the process of acquired resistance but that there are other undetermined factors which play a contributary, and at times a preponderating rôle.

It has been found repeatedly that active immunity may occur in the absence of demonstrable serum immune properties. Cecil and Blake (9) failed to find any constancy in the appearance of protective substances in the blood of monkeys recovering from pneumococcus pneumonia or any correlation between the degree of active immunity of the animal and the passive immune properties of its serum. Wright (10), studying experimental pneumococcus infection in rabbits, observed that the development of opsonic and protective properties was irregular and uncertain and did not appear to parallel the acquisition of active immunity. Other authors have made analagous observations.

This inconstancy in the finding of immune bodies in the serum of animals with acquired resistance appears to depend to some extent on the type of invading pneumococcus. While Cecil and Blake found protective substances occasionally after infection with Types I and II, none were observed to occur in the serum after pneumonia due to Pneumococcus Types III and IV. Differences in antibody response to the several pneumococcus types are brought out more clearly by active immunization. In contrast to the comparative ease and regularity with which it is possible to produce immune substances against Types I and II in the different animal species is the uncertainty of antibody response following immunization with Type III. In a recent study Tillett (11) found that only four of twenty-eight rabbits immunized with Type III pneumococcus showed type-specific antibodies in the serum. Tudoranu, however, considers that the difference between immune sera Types I and II and serum Type III is one of antibody concentration only and if enough serum of a Type III immunized or recovered animal is used, specific protection can be demonstrated against the homologous pneumococcus. Felton (12) has thrown further light on this subject by finding that in antipneumococcus serum or antibody solution there is an inhibitory or antagonistic substance which can be eliminated by fractional precipitation. He was able to show that a Type III antiserum thus treated had a much augmented protective power.

The appearance of serum immune bodies appears to be conditioned even more by the kind of animal employed in the study, than by the type of pneumococcus used. Those authors who have found opsonic activity constantly in the serum of animals recovering from pneumococcus infection (Bull and Tchistovitch) worked with a highly resistant species, the dog. Wadsworth, studying the problem, failed to find increased phagocytic power in dogs' blood regularly, but he never found any evidence of this reaction in infected rabbits. The majority of those workers whose findings have lead them to question or reject the probable causal relationship between the occurrence of humoral immunity and recovery have worked with animals highly susceptible to pneumococcus, chiefly rabbits and monkeys.

Among the experimental data of the above authors on pneumococcus infection in susceptible animals one finds occasionally suggestions that there may be some relationship between the degree of active immunity and the presence of demonstrable immune properties. Wright, for example, found evidences of humoral immunity, especially the pneumococcidal power of the blood, most constant and most marked at a time when the resistance of the animal (rabbit) was greatest. More recently Stillman (13) made the observation that rabbits exposed repeatedly to sprayings with Pneumococcus Type 1 developed protective properties in their serum, the percentage incidence of which increased progressively with the number of sprayings. Since the per cent mortality among the rabbits diminished with the number of exposures to pneumococci it is highly probable that the surviving animals had developed an increased resistance.

In taking up the present work, namely an attempt to determine the part played by humoral immunity in the mechanism of recovery from experimental pneumococcus infection, it seemed wisest to investigate first the reaction of resistant animals since they have been found to yield more constant demonstrable evidence of antibody production; then in the light of the findings which might result from such a study to proceed to observations on a susceptible species.

## *Methods.*

*Disease Production.*—Cats and rabbits were infected, for the most part by the intrapleural injection of actively growing and abundant broth cultures of pneumococci. A few animals were injected intraperitoneally. The organism used principally was a Type 1 pneumococcus originally isolated from the blood of a case of lobar pneumonia. After passage through twelve cats its virulence for these animals was found to lie between 0.005 cc. and 0.1 cc. broth culture. Rabbits were killed in the amounts of 0.000,000,1 cc. and mice with 0.000,000,01 cc. Passage through a cat was repeated every month or two during the course of the work. A Type II and a somewhat less virulent Type I pneumococcus were

also used in rabbits. The former killed in doses of 0.1 cc. to 0.01 cc., the latter with 0.000,01 cc. to 0.000,001 cc. broth culture.

*Disease Course.*—The cats which recovered ran a febrile course usually from 4 to 5 days terminating often with an abrupt fall in temperature. Occasionally the fever persisted for a longer period. A well marked loss in weight was a constant accompaniment. This usually ceased with the fall in temperature but in some animals there was a slight further weight loss followed by a slow subsequent gain, in spite of their appearing otherwise well. The course of infection in rabbits was much more irregular and fluctuations in temperature were found to be less significant than loss in weight. The experimental animals were observed from weeks to months.

*Pathology.*—The lesions produced in cats by intrapleural inoculation were found to vary widely. Autopsy after fatal termination of the infection showed, in the majority of instances, empyema of one or both sides often with pericarditis and sometimes peritonitis. In only one case was lobar pneumonia observed. Several animals showed no gross pathology, but blood cultures were positive. Some of the cats were killed during and immediately after recovery. Except in one instance in which organized fibrin was observed over the pericardium, no gross lesions were found in these animals. Cats dying after intraperitoneal injection showed peritonitis but no pleuritis. In the rabbits which died, a diffuse peritonitis and pleuritis were found. Lobar pneumonia was not seen. Microscopical studies of the tissues were not made except to verify the presence of pneumonia. Secondary infection with streptococci or Gram-negative bacilli supervening either during the course of infection or after recovery was not uncommon.

*Serum.*—The test serum was allowed to separate in the cold, then cleared by centrifugation and immediately inactivated at 56° for ½ hour. All specimens from a single animal, kept on ice in sealed tubes, were tested at one time. The dilutions indicated in the tables represent final dilution in the serum-leucocyte mixture.

*Pneumococcidal Tests.*—The serum of the infected animals was tested for its power to promote pneumococcus killing by adding it to normal rabbit serum-leucocyte mixtures seeded with virulent pneumococci. The technique was the same as that described in a previous paper in the study of this property of specific antipneumococcus serum (14). The infected animals' leucocytes were not used because it was not possible to obtain sufficient cells for the tests without withdrawing injuriously large quantities of blood and in the experiments with cats, normal leucocytes could not be employed since it would have been necessary to add normal active cat serum which mixture possesses pneumococcus-killing power. Heating the serum annuls this action. By using the serum-leucocyte mixture of a normal susceptible animal which of itself has no pneumococcidal action, any degree of this property occurring can be attributed directly to the effect of the added infected animal's serum. The amount of pneumococcus suspension

with which the serum-leucocytes were seeded was chosen arbitrarily as containing a sufficient number of pneumococci to produce abundant macroscopic growth in 15 to 18 hours but still few enough to permit effective action of the immune serum in high dilution.[1]

*Opsonic and Agglutination Tests.*—The serum was tested for opsonins and agglutinins according to the technique described in a preceding paper (3). The ratio of serum to pneumococcus suspension employed was 20:1.

## EXPERIMENTAL.

### *Cats.*

### *Development of Pneumococcidal Promoting Properties in the Serum Following Infection and Their Relation to Active Immunity.*

It was found constantly that the serum of cats recovering from experimentally induced pneumococcus infection possessed the power to promote destruction of pneumococci in rabbit serum-leucocyte mixtures analagous to that caused by the presence of specific anti-pneumococcus serum. This property or substance was heat-stable and could be demonstrated in high dilutions of the serum. The method of testing such sera is shown in Table I in which are given the details of the tests made on the serum specimens of Cat 1, Text-fig. 1, secured before and after infection. It is seen that the presence of inactivated normal cats' serum had no detectable retarding effect on the growth of pneumococci in rabbits' serum and leucocytes even in dilution of 1:10 (lower dilutions could not be used on account of the disturbing effect of cytotoxic action), whereas the serum of the same cat obtained after recovery from a well marked infection, conferred pneumococcus-killing power on these mixtures in dilution as high as 1:5120. The immune horse serum tested at the same time as a control on the activity of the rabbit serum and leucocytes showed a potency ten times as great.

Following recovery the cats were found to be resistant to many times the fatal dose of pneumococci.

[1] In a previous paper (14) it was found that there exists a quantitative relationship between the concentration of immune serum present and the number of pneumococci destroyed by the serum and leucocytes.

*Appearance of Serum Immune Properties in Relation to Temperature and Blood Invasion.*

Tests on samples of blood taken frequently during the course of infection showed that in those animals destined to recover, serum immune properties appeared about the 4th day of the disease (Text-figs.

TABLE I.

*Determination of Pneumococcidal Promoting Power of Cat's Serum before and after Pneumococcus Infection.*

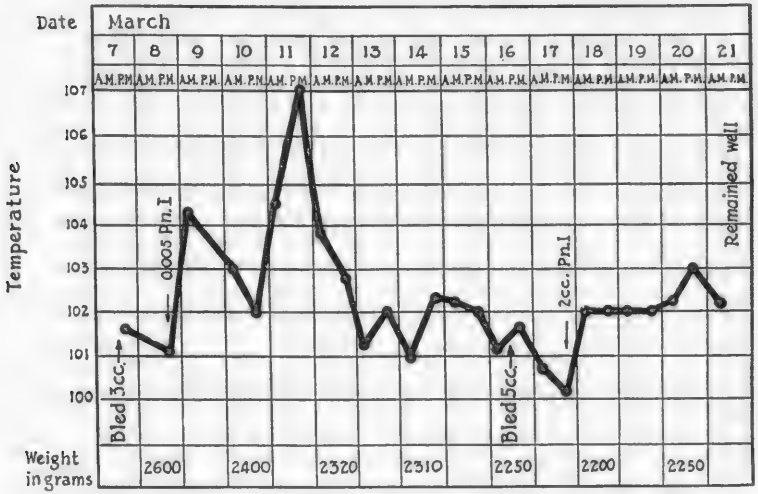
Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + diluted cat serum 0.1 cc. + pneumococcus suspension 0.1 cc.

| | Amount of standard pneumococcus suspension | Dilutions of cat serum | Growth as shown by color change at hrs.* | | | | Pneumococci in stained film at 72 hrs. |
|---|---|---|---|---|---|---|---|
| | | | 15 | 18 | 24 | 48 | |
| | *cc.* | | | | | | |
| Cat's serum before inoculation | 0.000001 | 1:10 | ++++ | | | | + |
| | " | 1:20 | ++++ | | | | + |
| | " | 1:40 | ++++ | | | | + |
| | " | 1:80 | ++++ | | | | + |
| Cat's serum after recovery | " | 1:320 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:640 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:1280 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:2560 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:5120 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:10240 | 0 | 0 | ++ | | + |
| | | Dilutions of immune horse serum | | | | | |
| Controls with immune horse serum | " | 1:6400 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:12800 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:25600 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:51200 | 0 | 0 | 0 | 0 | 0 |
| | " | 1:102400 | 0 | 0 | +++ | | + |
| Controls with normal rabbit serum and leucocytes only | " | | ++ | +++ | | | + |
| | " | | +++ | ++++ | | | + |
| | 0.0000001 | | + | +++ | | | + |
| | " | | +++ | ++++ | | | + |
| | " | | +++ | ++++ | | | + |

* Degree of methemoglobin formation.

2, 5, and 6). In two cases they were demonstrable on the 3rd day. The titer of the pneumococcidal promoting substances rose abruptly and usually reached its highest point within 48 hours where it tended to remain comparatively stationary for several days before beginning a gradual drop. Coincident with the appearance of immune bodies in the serum there occurred in most instances a sharp drop in temperature, although often it did not reach normal until several days later.

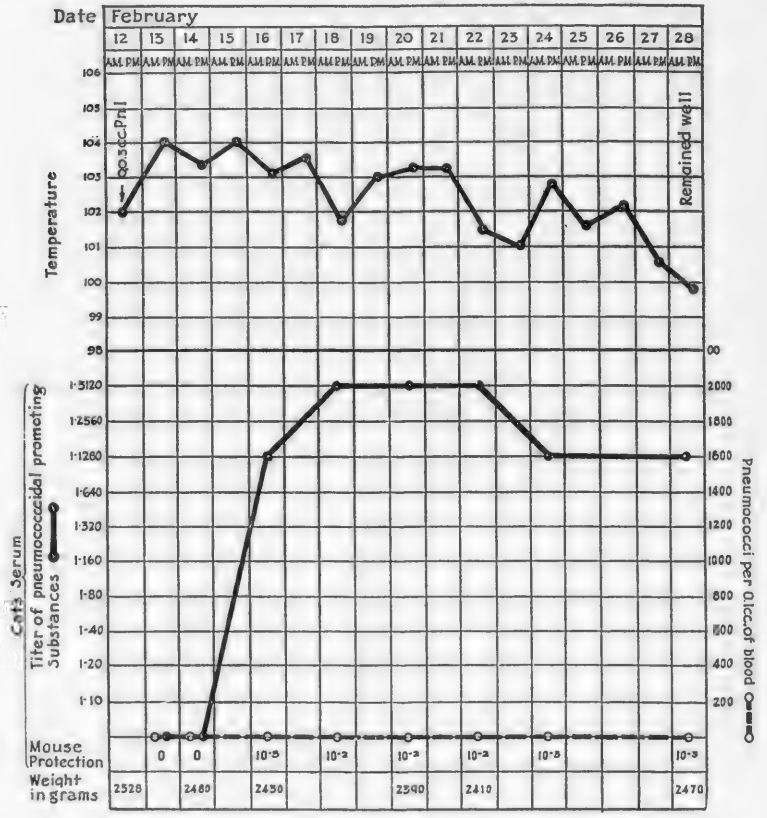
Blood invasion which was present in the majority of the infected cats and often to an intense degree, terminated abruptly with the




TEXT-FIG. 1. Cat 1. Experimental pneumococcus infection following the intrapleural injection of 0.005 cc. of Pneumococcus Type I broth culture.

appearance of pneumococcidal promoting substances in the blood (Text-figs. 3, 5, and 6). In most instances this immune property was not demonstrated until the blood was sterile, but occasionally as shown by Cat 5, Text-fig. 5, both antipneumococcus substances and pneumococci were found to be free in the blood stream at the same time. It is possible that had blood specimens been taken with sufficient frequency this transient simultaneous presence of circulating

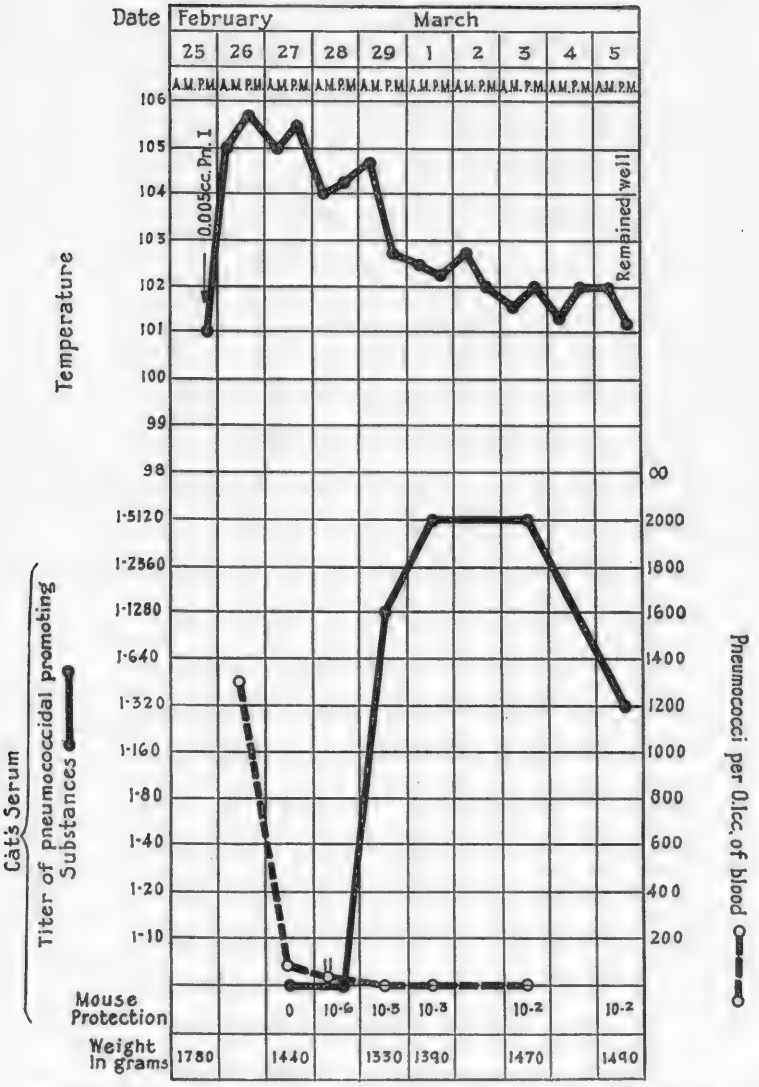
antigen and antibody would have been observed much more frequently or perhaps regularly. The constant and marked decrease in the number of colonies shown by blood plates during the 24 to 48 hours



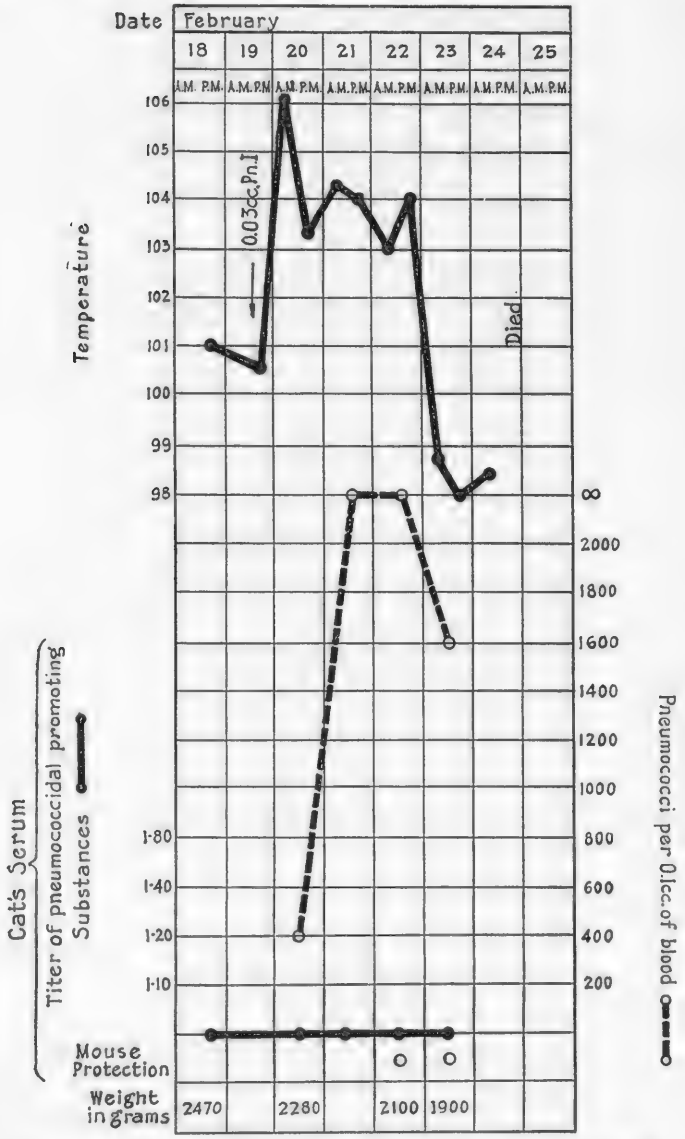

TEXT-FIG. 2. Cat 2. Experimental pneumococcus infection following the intrapleural injection of 0.03 cc. broth culture, Pneumococcus Type I.

preceding the appearance of immune substances in the blood, suggests that the elaboration of these bodies begins early in the disease.

All those cats in which the disease went on to a fatal termination

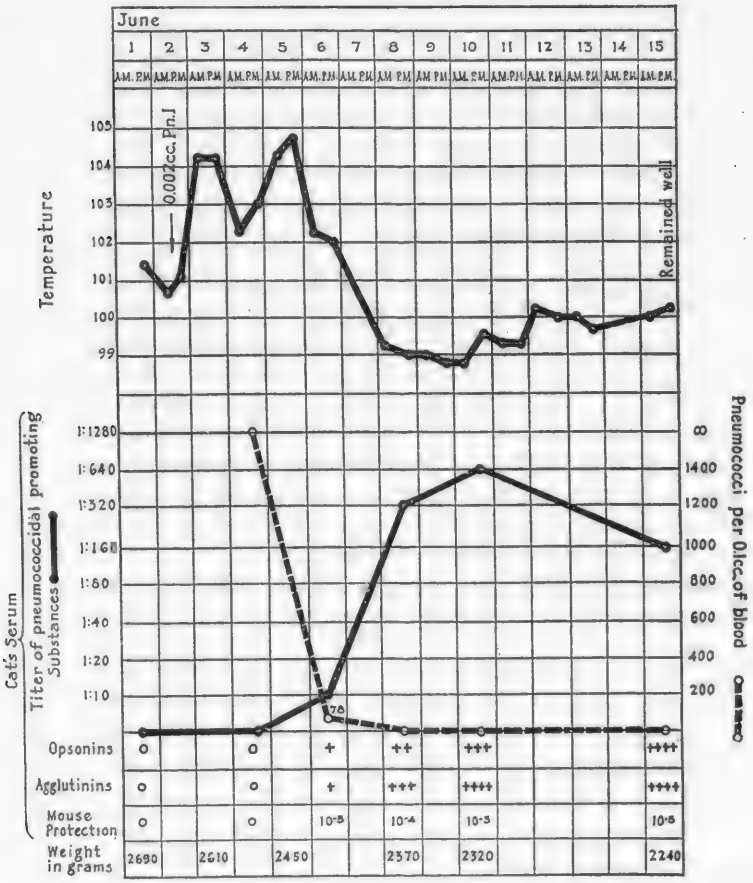



TEXT-FIG. 3. Cat 3. Experimental pneumococcus infection following the intrapleural injection of 0.005 cc. broth culture, Pneumococcus Type I.




TEXT-FIG. 4. Cat 4. Experimental pneumococcus infection following the intrapleural injection of 0.03 cc. broth culture, Pneumococcus Type I.

failed to develop demonstrable serum immune properties and showed a persistent marked bacteriemia (Text-figs. 4 and 7).


June
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A.M. P.M.
0.002 cc. Pn.I
Temperature
105 104 103 102 101 100 99
Remained well
Cat's Serum
Titer of pneumococcidal promoting Substances
1:1280 1:640 1:320 1:160 1:80 1:40 1:20 1:10
Pneumococci per 0.1 cc. of blood
8 1400 1200 1000 800 600 400 200
Opsonins 0 0 + ++ +++ ++++
Agglutinins 0 0 + +++ ++++ ++++
Mouse Protection 0 0 10-5 10-4 10-3 10-5
Weight in grams 2690 2610 2450 2570 2320 2240

TEXT-FIG. 5. Cat 5. Experimental pneumococcus infection following the intraperitoneal injection of 0.002 cc. broth culture, Pneumococcus Type I.

*Character of Immune Bodies Occurring at Recovery.*

In a number of instances serum specimens were also tested for their opsonic, agglutinative, and mouse protective actions. The results of

one such complete study are shown in Text-fig. 5. A very close parallelism was found to exist between the appearance of these three well recognized evidences of antipneumococcus reaction and the pneumococcidal promoting power of the serum. With very few exceptions they all appeared at the same time and increased together although not necessarily in a strictly quantitative relationship. In two cases mouse protective properties[2] were demonstrable before pneumococcidal promoting power became apparent. Text-fig. 3 shows one such instance. A repetition of the test gave the same result. And in one other cat the serum showed slight opsonic activity the day preceding the detection of the other evidences of immune change. The absence of exact parallelism between the appearance and rates of increase in intensity of these several reactions affords, however, no objection to the supposition that they represent different manifestations of one immune response since the various methods employed to bring them out are not comparable quantitatively. During the initial period of recovery when the concentration of immune substances was low, frequently neither agglutinative nor opsonic activity could be demonstrated with equal parts of serum and pneumococcus suspension but could be brought out clearly when a ratio of twenty parts serum to one part pneumococcus suspension was used.

The animals which died failed to show at any time during the course of the disease any of the above studied serum immune properties.

*Specificity of Serum Immunity.*

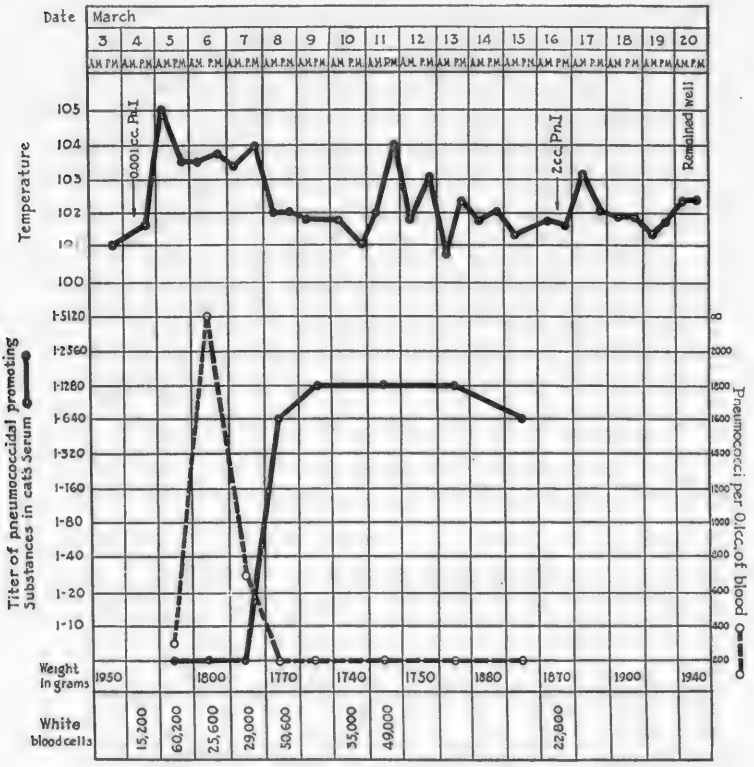
Tests carried out to determine the specificity of the immune response in the serum of recovered cats showed that such serum was strictly type-specific as to pneumococcidal promoting, agglutinative, and opsonic properties.

*Relation of Leucocyte Count to Course of Disease.*

Estimation of the number of circulating white blood cells made at frequent intervals during the experimental disease, failed to reveal any constant difference in leucocyte response between the animals recovering from infection and those which succumbed. After an

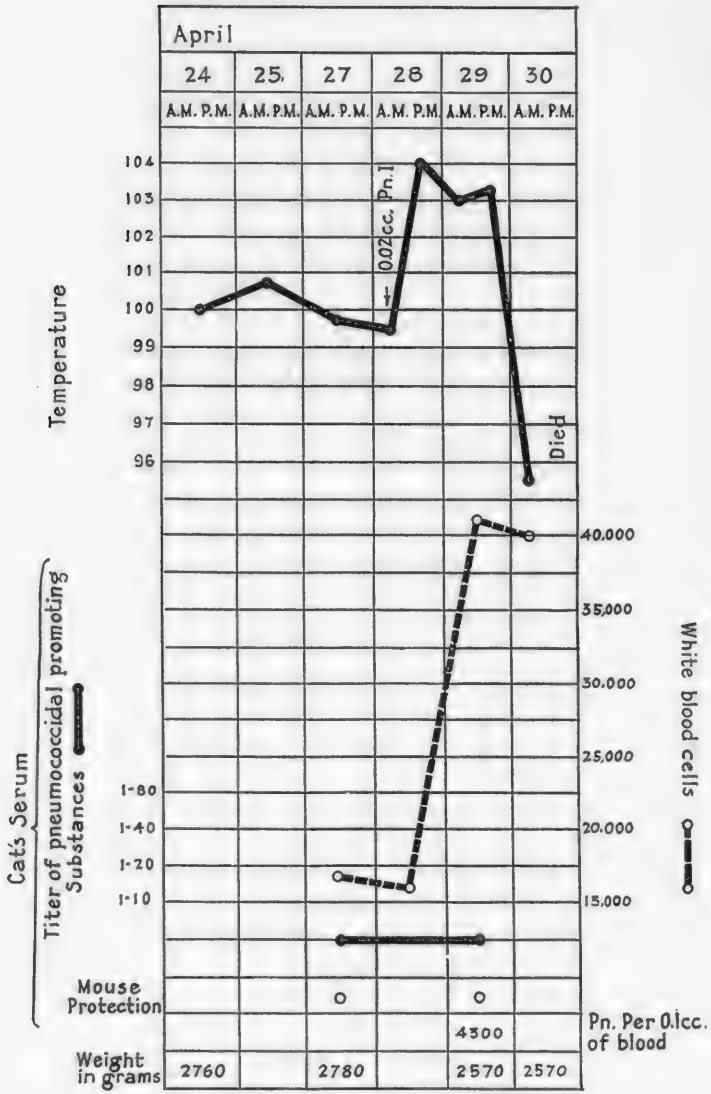
[2] The figures recorded on the charts indicate the dilution of culture against which 0.2 cc. of the animal's serum protected.

initial rise within the first 24 to 48 hours from a normal count of 10,000–15,000, to 30,000–50,000, the number of leucocytes usually diminished gradually during the course of the disease. In a few instances a secondary rise was noted just preceding recovery. In sev-




TEXT-FIG. 6. Cat 6. Experimental pneumococcus infection following the intrapleural injection of 0.001 cc. broth culture, Pneumococcus Type I. Reinoculated after recovery with 2 cc. broth culture.

eral cats both with fatal and non-fatal outcome the number of white blood cells increased only slightly. It was of interest to observe repeatedly an unusually high leucocyte count accompanying fatal




TEXT-FIG. 7. Cat 7. Experimental pneumococcus infection following the intraperitoneal injection of 0.02 cc. broth culture, Pneumococcus Type I.

infection (see chart of Cat 7, Text-fig. 7). Another cat (chart not exhibited) showed a count rising from 33,000 on the 1st day to 50,000 on the 4th day when death occurred. Still another died on the 8th day with the white blood cells at 42,000. Counts as high as 85,000 were observed within 2 or 3 days of death. In certain of these animals showing a terminal high white count empyema was found, but in others there was no evidence of complications.[3]

It is evident from these observations that the course of pneumococcus infection in the cat is not determined primarily by the number of circulating leucocytes. In the absence of detectable serum immunity the leucocytes, no matter in how great numbers present, seem to have no inhibiting effect on either blood invasion or progression of the disease. Cat 6, Text-fig. 6, showed a rapidly increasing number of pneumococci in the blood in spite of a leucocyte count of 60,000, and it was not until humoral immune bodies appeared that blood invasion ceased. It was noted repeatedly that with immune substances present in the blood a moderate increase only in the number of leucocytes appeared to be compatible with rapid and complete recovery.

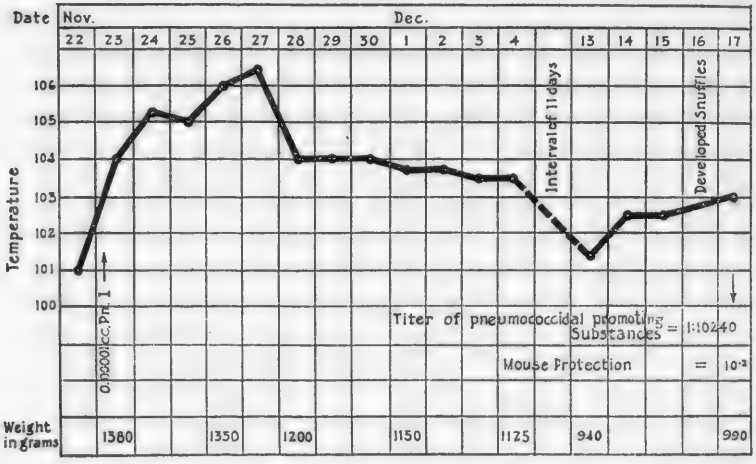
While the scope of this work did not include a complete study of the duration of active and passive immunity following infection, observations were made on three cats after a lapse of 2 months. Just prior to reinoculation with 10 to 20 times the killing dose of pneumococci two of the cats showed a low titer of pneumococcidal promoting substances in the serum, the third showed none. All three animals reacted with a marked rise in temperature and weight loss but recovered in 3 to 4 days. In striking contrast to the behavior of these animals is the absence of any appreciable reaction observed in cats reinoculated soon after recovery from infection when the concentration of serum immune substances was at a high level (see charts, Text-figs. 1 and 6). These were two of the cats used for the tests 2 months later.

*Rabbits.*

Attempts to study in rabbits the type of experimental pneumococcus infection observed in cats were found to be attended by many

[3] Furthermore the development of empyema was not always accompanied by a rise in the number of leucocytes.

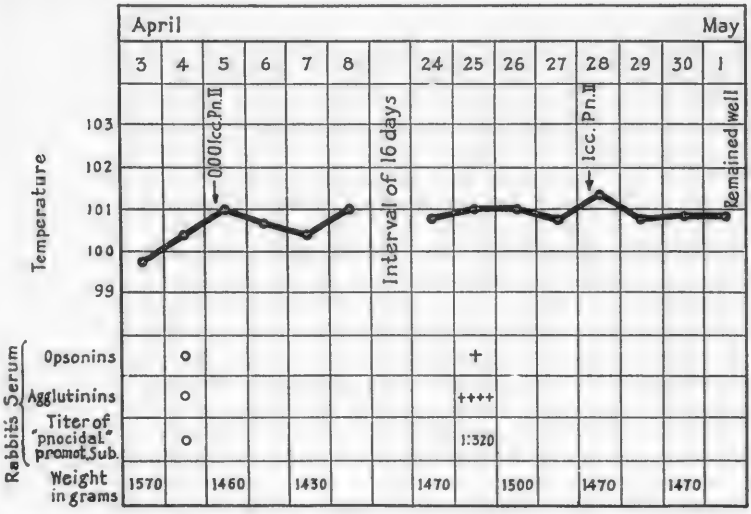
difficulties on account of the wide individual variations in susceptibility which rabbits manifest towards the pneumococcus. A great majority of the animals inoculated, either died or failed to show evidence of disease. Furthermore, withdrawal of blood in any quantity during the stage of active infection had very disturbing and often disastrous effects. From a very large number of trials a few satisfactory observations were secured but these were by no means as complete as those made on cats.




TEXT-FIG. 8. Rabbit 1. Experimental pneumococcus infection following the intrapleural injection of 0.000,01 cc. broth culture, Pneumococcus Type I.

The main findings in cats as regards the occurrence of immune substances in the blood, were confirmed in the rabbit. Following recovery from experimental pneumococcus infection, there appeared in the rabbit's serum pneumococcidal promoting substances which were shown to be associated with the acquisition of greatly increased pneumococcus resistance (Text-figs. 8, 9, and 10). Likewise opsonic, agglutinative, and mouse protective properties were demonstrable in the serum at the same time. The intensity of the immune response appeared to be fully as great in rabbits as in cats when similar experimental conditions obtained.

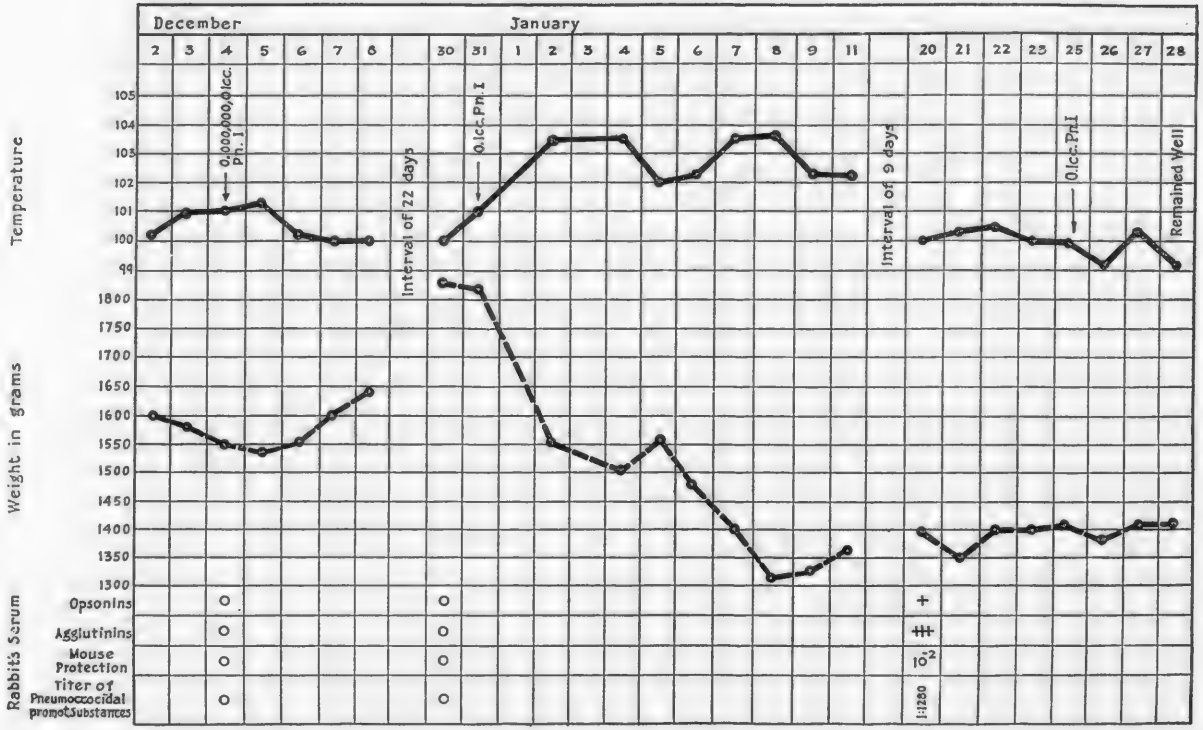
The charts of the three rabbits exhibited exemplify several types of reaction to pneumococcus infection which differ considerably one from the other. Rabbit 1, Text-fig. 8, received a small dose of a virulent strain and after a severe and prolonged disease course showed a high concentration of serum immune substances.[4] Rabbit 2 after a much larger dose of a pneumococcus of considerably less virulence, gave scarcely any evidence of disease but showed a well defined though low titer of pneumococcidal promoting power in the serum. With




TEXT-FIG. 9. Rabbit 2. Experimental pneumococcus infection following the intraperitoneal injection of 0.001 cc. broth culture, Pneumococcus Type II. Reinoculation after recovery with 1 cc. broth culture.

Rabbit 3, on the other hand, the injection of a minute number of highly virulent pneumococci, produced no appreciable effect and no resulting detectable serum immunity but the animal was found to have developed a very greatly increased resistance. While Rabbit 2 with demonstrable serum immune properties showed no reaction

[4] Further observations were prevented by the development of snuffles which progressed slowly to a fatal termination.


December
January
2 3 4 5 6 7 8 30 31 1 2 3 4 5 6 7 8 9 11 20 21 22 23 25 26 27 28
0.000,000,01cc. Pn. I
Interval of 22 days
0.1cc. Pn. I
Interval of 9 days
0.1cc. Pn. I
Remained Well
Temperature
105 104 103 102 101 100 99
Weight in grams
1800 1750 1700 1650 1600 1550 1500 1450 1400 1350 1300
Rabbit's Serum
Opsonins
Agglutinins
Mouse Protection
Titer of Pneumococcidal promot. Substances
o o o o o o
+ ++ 10^2 1:1250

TEXT-FIG. 10. Rabbit 3. Experimental pneumococcus infection following the intraperitoneal injections of 0.000,000,01 cc. broth culture, Pneumococcus Type I, on December 4, and 0.1 cc. on December 31. Reinjection of 0.1 cc. on January 25.

following the injection of many times the lethal dose of culture, Rabbit 3, lacking such serum properties, developed a severe though not fatal disease after reinoculation. The subsequent appearance of immune substances in the serum of Rabbit 3 indicated a further increase in resistance as revealed by the lack of any detectable reaction to a second reinoculation of the same size.

Observations on the leucocyte count were not made in the rabbits chiefly on account of the uncertainty of the outcome of the experimental disease. Neither were the rabbits retested for duration of their active or passive immunity.

## DISCUSSION.

The most noteworthy result of this work is the constancy of the findings. In the resistant animal recovery was always marked by the development of well defined humoral immune properties which failed to occur in fatal infections. In the susceptible animal studied (the rabbit) similar immune reactions were found although they could not be related so closely to the period of recovery. The observations in cats tend to amplify the studies of certain previous investigations on experimental pneumococcus infection in resistant animals. Bull (4), who has made the most detailed report on this subject, was not able to demonstrate agglutinins and opsonins in the serum of dogs until 24 to 48 hours after the temperature had fallen, but he found indirect evidence of immune body action *in vivo*, at the time of crisis. Whether the success obtained in our experiments in demonstrating the coincidence of the appearance of serum immune substances with recovery was due entirely to the methods employed or to the particular experimental animal used remains to be determined.

Our findings in the rabbit do not agree altogether with those of other investigators. The results obtained in the study of Rabbit 3, Text-fig. 10, offer a possible explanation for at least some of these previously observed irregularities in the appearance of immune substances in susceptible animals. This animal, as a result of an exceedingly small injection of a highly virulent pneumococcus developed a marked degree of active immunity but showed no evidence of passive immune properties in its serum. Is this to be attributed to the presence of immune substances in such low concentration that they could

not be detected in the test-tube or mouse, or were the body cells only sensitized and ready to react more energetically to further antigenic stimulus, or was this state of resistance due to another factor? The finding of a relatively high concentration of serum immune substances following the severe infection produced by the second inoculation suggests the first or second possibility. Another factor which may introduce variations in the demonstrability of humoral immune bodies is possible differences among the susceptible animal species in respect to excess elaboration of antipneumococcus substances. Cecil and Blake (15), for example, found only very occasionally slight traces of immune properties in the serum of monkeys tested 2 to 3 weeks following experimental pneumococcus pneumonia with all four types.[5] It seems not improbable that certain non-resistant species, as the monkey, may elaborate specific antibodies in only slight excess of the concentration needed to combat the invading microorganism and that this detectable excess disappears rapidly after recovery. Dochez (16) observed in certain cases the rapid disappearance of protective substances from the serum of patients after recovery from lobar pneumonia. We have also noted, following lobar pneumonia, the complete disappearance within 10 days of serum immune substances which were present at the time of crisis in considerable concentration.[6]

The chief significance of our observations on the occurrence of antipneumococcus immune substances in the cat would seem to lie in the time relation of their appearance to the termination of infection and the nature of their demonstrable action. The fact that substances capable of promoting a marked degree of pneumococcidal action appeared in the serum coincidentally with the amelioration of the disease process as shown by drop in temperature, and cessation of blood invasion which was followed by continued progress to recovery, makes it seem probable that the development of humoral immunity bears a causal relationship to the mechanism of increased antipneumococcus defense. A perfectly valid criticism of this assumption is that we have tested only the animals' serum and that we have no

[5] That the monkey shows a well marked antibody production under certain conditions was found by Cecil and Blake (15) in their studies in vaccination with virulent living pneumococci.

[6] This finding will be reported in a subsequent study on lobar pneumonia.

evidence that the serum of the recovering animal promotes the killing of pneumococci in the cat's body as it does in the test-tube with normal leucocytes. It is true that we have made no observations on the phagocytic and intracellular digestive activity of the leucocytes during experimental pneumococcus disease. The studies of other workers (17, 18, 19), however, on the activity of the leucocytes during pneumonia, while not in entire agreement with each other, indicate that ordinarily their function is little if any diminished, but in infections of marked severity there may be some depression especially of the intracellular digestive power. This phase of the problem requires further investigation.

The essential change which occurs in the serum of the cat recovering from pneumococcus infection would seem to be the elaboration of a heat-stable body or bodies whose chief antibacterial manifestations are opsonic and agglutinative activity. Power to precipitate the soluble substance undoubtedly is also present since the immune change is specific. That all these reactions are brought to bear on the pneumococcus growing in the animal body seems probable. Whether these several functions of the immune serum assume the same relative importance in the test-tube serum-leucocyte mixture is open to question, since conditions of pneumococcus growth *in vivo* differ in some respects from those *in vitro*. Possibly the precipitative action plays a less important rôle in the serum-leucocyte tests than in the body where the products of pneumococcus growth are present in greater concentration. Again, agglutination may play a more prominent part *in vivo*, especially in freeing the blood stream from organisms. However, the fact that the potency of an immune serum to produce pneumococcus killing in the serum-leucocyte mixtures parallels very closely its protective power in the mouse's body (14), suggests that its chief function is the same in both environments. Since phagocytosis and intracellular digestion are promoted to a marked degree by the presence of antipneumococcus serum and constitutes the only means of pneumococcus killing by the blood elements which we have been able to demonstrate, we are lead to infer that opsonic action in its entirity probably constitutes the most important property of such a serum.

The question then arises whether granting the possibility of de-

struction of pneumococci in the body by the above indicated means, will account for the degree of pneumococcus killing that occurs during recovery from infection. This question cannot be answered definitely since we have no means of estimating the number of organisms in the infected animal, but it is possible to estimate in a general quantitative way the killing power of immune blood. As shown in Table I, the immune horse serum is ten times as potent as the recovered cat serum. By repeated tests we have found that a 1 to 500 dilution of immune serum of this potency when added to the serum and leucocytes contained in 0.5 cc. normal blood is capable of causing the destruction of $10^{-3}$ of the standard pneumococcus suspension which amount contains approximately 1,000,000 pneumococci (14). With a serum one-tenth this potency we might expect that it would produce the same action in a dilution of 1 to 50, since the action of immune serum under these conditions has been found to be quantitative in nature. A cat weighing 2000 gm. would have a total blood volume of about 100 cc. and if each 0.5 cc. of blood could destroy 1,000,000 pneumococci the total amount disposed of by 100 cc. would be 200,000,000. But in making this estimate we are limiting the destruction to the normal number of circulating leucocytes. The serum is capable of sensitizing perhaps ten times or more as many organisms as can be cared for by the normal number of circulating white blood cells and it is likely, as shown by the work of others which will be discussed further on, that certain of the fixed tissue cells also take part in the phagocytosis under these conditions. Even if these figures, which are only approximate, err considerably on the side of too high an estimate they do show a relatively enormous killing potentiality in the serum and cells of the pneumococcus immune animal, and it is probable that this process occurs much more effectively and continuously in the body than in the test-tube.

The relation of the naturally immune state to these changes which have been found to take place during infection in the cat, is by no means clear. Assuming that the normal resistance of this animal depends chiefly on the presence of circulating antipneumococcus opsonins, it would seem probable that a pneumococcus sufficiently virulent to establish itself in a free cavity of the body must be able to

neutralize the normal immune substances.[7] What happens next we can only surmise. This process of neutralization may take place rather slowly or it may be that the body has reserves of normal defense forces that can be called into action to retard the growth of the pneumococci until the development of the acquired immune reaction occurs. Wright considers that increased resistance may begin within a few hours after the introduction of the microorganisms and we also have reason to believe that the processes of acquired immunity are initiated relatively early in the disease course. The newly operating anti-pneumococcus opsonins detectable at recovery appear to differ in nature from the normal opsonins but we have as yet insufficient criteria to be sure of this. Our observations in a previous paper (3) on the relative heat resistance of normal pig opsonins raises the question as to the safety of relying entirely on this test for the differentiation of immune from normal opsonins. At any rate the pneumococcidal action induced by the recovering cat serum in a rabbit serum-leucocyte mixture appears to be the same as that caused by the serum and leucocytes of the normal cat, but whether the pneumococcus-killing process which takes place in the animal successfully resisting infection by implanted pneumococci is the same as that employed by the diseased animal is open to question. Information concerning the type of cell engaged in phagocytosis and intracellular digestion of pneumococci in naturally resistant animals is far from complete. The evidence at hand, however, suggests that of the circulating leucocytes, the polymorphonuclears play the chief rôle in this process. The large monocytes have also been shown to be actively phagocytic for pneumococci. Observations in animals with acquired immunity indicate that a variety of cells take part in the disposal of pneumococcus.

Wright, studying the fate of pneumococci injected into rabbits immunized against Pneumococcus Type I found that in the lung the monocytes were fully as actively phagocytic as the polymorphonuclear leucocytes and that in the liver certain of the fixed tissue cells of the reticulo-endothelium engulfed large numbers of organisms. Singer

[7] Bull (4) regards the early stages of disease as a period of adaption of the microorganism to the existing antibodies rather than to the exhaustion of these substances.

and Adler ascribe phagocytosis in both the immune and normal animal entirely to the reticulo-endothelium. However, they did not demonstrate this type of phagocytosis as occurring in the normal animal. Tudoranu found that in the aleuronat peritoneal exudate of rabbits immunized with Pneumococcus Type III the homologous organisms were phagocyted almost entirely by the monocytes. The experiments of Winternitz and Kline (20) and also Wright in which the number of leucocytes were greatly reduced by benzene injections without appreciably diminishing the blood-clearing power of the immune animal, indicate that cells other than the leucocytes are active in the pneumococcus-destroying processes of the immune body. While all these experimental observations have been made in a susceptible animal, the rabbit, the same process may well occur in relatively resistant animals with acquired immunity and, were it possible to make observations in the normal animal body as easily as in the immune animal, cells, other than the leucocytes, might be found to be active in the destruction of pneumococcus. That the Kupffer cells of the liver in normal resistant animals are capable of taking up pneumococci seems quite probable but it is difficult to estimate the importance of this process in the normal defense mechanism against natural infection since we know practically nothing of the penetration of pneumococci into the blood stream except during disease.[8]

The observations on rabbits recorded in this study provide by themselves only indirect evidence as to the part played by humoral immunity in the rabbit's recovery from pneumococcus infection. But taken in conjunction with the findings in cats, they assume much greater significance. However, the fact should not be overlooked that acquired resistance in this animal has been shown to be present in the absence of demonstrable serum immunity and although certain plausable explanations for such absence can be made, there exists the possibility of other unknown immune factors.

It should be stressed that our experiments both with normal and infected animals have been carried on almost entirely with pneumococci of Types I and II. That the blood of pneumococcus-resistant

[8] Kyes demonstration of the marked phagocytic power of the Kupffer cells of the pigeon for pneumococci suggests that these cells in the normal mammal may possess a similar function.

animals possesses the power to destroy Pneumococcus Type III (1) and that it possesses normal opsonins against this type (21), has been shown. But no experiments on infection with Type III pneumococci nor studies on normal resistance of susceptible animals against these organisms have been made. Tillett (22) has recently published a study which might be interpreted to indicate that natural and acquired immunity to Pneumococcus Type III may be of a different order than that against the other two fixed types. He found that the resistance of normal rabbits to relatively enormous intravenous doses of an encapsulated Type III pneumococcus was not associated with demonstrable antibodies in the serum and that little evidence of phagocytosis could be detected in the blood. Phagocytosis by the reticulo-endothelial system was not investigated. The most significant phase of Tillett's work deals with the production of active immunity to Type III which he finds to be not only not associated with demonstrable immune substances but to be non-specific. He considers that the normal rabbit possesses a mechanism for inflicting injury on the capsule of the Type III pneumococcus and that active immunity probably consists in an increase in this normal function.

In the light of our present incomplete knowledge it is not possible to draw any general conclusions concerning the processes underlying recovery from experimental pneumococcus infection. Until the whole field has been studied with the care that has been devoted to certain areas, the interrelationship of the component parts will probably not be revealed. However, the constant occurrence of phenomena observed in the study of certain aspects of the problem justify our drawing tentative inferences limited always to the conditions under which the observations were made. Thus the evidence at hand would indicate that recovery from experimental infection with Pneumococcus Types I and II in cats and rabbits is brought about largely by the elaboration of specific antipneumococcus serum substances whose chief function is to make possible phagocytosis and intracellular digestion of the invading pneumococci. That the evidence for this assumption is much clearer in cats than in rabbits is granted. Furthermore drawing the above conclusion does not exclude the possibility of other forces aiding in the process. There may be another factor in acquired immunity and certain experimental findings suggest its

existence, which plays a part perhaps relatively more important in one type of animal than another and possibly varying in its activity with the different types of pneumococci. That other pneumococcus-resistant animals react in a manner similar to the cat seems probable in view of observations of other investigators and because of the fact that these animals show the same type of normal protective mechanism. Inferences concerning the recovery processes of pneumococcus-susceptible animals other than the rabbit are less safe because of the great diversity of findings by different workers. However, the fact that humoral immune substances have been detected in these animals with acquired resistance, suggests that this form of immunity plays some part in their recovery from pneumococcus infection.

## SUMMARY.

A study was made of the blood of cats and rabbits during experimental pneumococcus infection with a view to ascertaining the relationship of acquired immune properties to the mechanism of recovery. Observations were directed chiefly towards the detection of pneumococcidal promoting substances, but the other manifestations of anti-pneumococcus reaction were studied as well. It was found constantly that the serum of animals recovering from infection possessed the power to promote the destruction of highly virulent pneumococci in rabbit serum-leucocyte mixtures which mixtures of themselves have no growth inhibitory action. Furthermore, the presence of this serum immunity was associated with a marked increase in acquired resistance to the pneumococcus. In cats which were studied in the most detail the pneumococcidal promoting power of the serum as well as the opsonic, agglutinative, and mouse protective activities became demonstrable at the time of recovery and their appearance in the serum always marked the termination of blood invasion. These immune reactions were found to be type-specific. The animals which succumbed failed to develop detectable serum immune properties and showed persistent blood invasion. The degree of leucocytosis did not appear to bear any constant relation to the outcome of the disease. The significance of these findings is discussed.

BIBLIOGRAPHY.

1. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xxxix, 219.
2. Woo, S. T., *J. Exp. Med.*, 1926, xliii, 623.
3. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1927, xlvi, 239.
4. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.
5. Tchistovitch, N., *Ann. Inst. Pasteur*, 1904, xviii, 304.
6. Tudoranu, G., *Ann. Inst. Pasteur*, 1926, xl, 606.
7. Singer, E., and Adler, H., *Z. Immunitätsforsch.*, 1924, xli, 71, 468.
8. Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.
9. Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 685.
10. Wright, H. D., *J. Path. and Bact.*, 1927, xxx, 185.
11. Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 713.
12. Felton, L. D., *Bull. Johns Hopkins Hosp.*, 1926, xxxviii, 33.
13. Stillman, E. G., *J. Exp. Med.*, 1927, xlv, 1057.
14. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xl, 467.
15. Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 65.
16. Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.
17. Tunnicliff, R., *J. Infect. Dis.*, 1911, viii, 302.
18. Rosenau, E. C., *J. Infect. Dis.*, 1906, iii, 683.
19. Eggers, H. E., *J. Infect. Dis.*, 1912, x, 48.
20. Winternitz, M. C., and Kline, B. S., *J. Exp. Med.*, 1915, xxi, 320.
21. Sia, R. H. P., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 709.
22. Tillet, W. S., *J. Exp. Med.*, 1927, xlvi, 343.